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ENDOCRINOLOGY

VOLUME 42 JANUARY, 1948 NUMBER 1

STUDIES ON THE BIOASSAY OF HORMONES
THE ASSAY OF TESTOSTERONE PROPIONATE AND ANDROSTERONE BY A CHICK COMB INUNCTION METHOD[1]

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PREVIOUSLY Frank et al. (1942) described a chick comb method for the assay of androgens. This communication is concerned with the application of this method to the assay of androsterone and testosterone propionate.

ANIMALS, METHODS, MATERIALS

White Leghorn male and female chicks obtained from the Kerr Chickeries, Frenchtown, New Jersey were employed in this study. They were shipped from the hatchery on the day of hatching and received in Cleveland the following day at the age of 1 to 2 days. The following day, at the age of 2 to 3 days, inunctions were started and continued once daily for 7 days. The total dose of androgens was applied to the comb in a total of 0.35 cc. of corn oil. The assays were carried out as described in detail by Frank et al. (1942).

The androsterone and testosterone propionate were kindly supplied by Ciba Pharmaceutical Products, Inc.

EXPERIMENTAL

Assay of Androsterone

Androsterone was assayed at levels of 10 to 40 micrograms and calculated according to the formulations of Frank et al. (1942). The

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results of 20 experiments and 6 groups of controls are represented in Table I. When the formula of Frank et al. (1942) was applied to the

TABLE I. THE ASSAY OF ANDROSTERONE BY THE METHOD OF FRANK ET AL. (1942)

Androsterone administered micrograms	Number of chicks used		Amount of androsterone recovered micrograms	Error %
	Males	Females		
0	9	11	4.4	—
0	7	13	4.2	—
0	8	11	6.4	—
0	8	11	6.0	—
0	5	12	6.0	—
0	11	8	4.4	—
10	14	5	16.9	+69
10	7	9	20.0	+100
15	8	11	8.2	−45
15	4	10	20.0	+33
20	10	9	18.4	− 8
20	9	11	22.0	+10
20	11	6	22.0	+10
20	13	7	21.2	+ 6
20	11	6	22.1	+11
20	7	10	23.4	+17
20	9	8	20.4	+ 2
20	6	13	19.5	− 3
25	11	9	24.4	− 2
30	11	9	27.3	− 9
30	14	5	21.3	−29
30	6	13	23.2	−23
30	8	10	33.3	+11
40	5	15	29.2	−27
40	7	13	32.3	−19
40	7	13	26.8	−33

controls, the values indicated a mean of 5.2 mg. of androsterone with a variation from 4.2 to 6.4 mg. for the individual groups. At the lowest dose studied, 10 mg., an overestimate was indicated with errors of +69 and +100 respectively while at the highest dosage level, 40 mg., there was an underestimate in the order of 19 to 33 per cent. Between the levels of 15 to 30 micrograms of androsterone there was a tendency for both positive and negative errors.

Assay of Testosterone Propionate

For the assay of testosterone propionate the essential details of the method were preserved but the initial body weights of the chicks were not considered. The final comb weights and the body weights were determined and the ratio of the comb weight in milligrams to the body weight in grams was used for the calculations. Both male and female chicks were employed.

Table II illustrates the responses to testosterone propionate from 5 mg. to 160 mg. All the data contained in this table were determined during one run. In order to determine the predictable error of the method one-half of the observations are considered as the standard

TABLE II. THE RESPONSE OF THE CHICK COMB TO TESTOSTERONE PROPIONATE

(Response expressed as ratio of comb weight in milligrams to body weight in grams)

5μg.		10μg.		20μg.		40μg.		80μg.		160μg.	
S♂	U♂	S♂	U♂	S♂	U♂	S♂	U♂	S♂	U♂	S♂	U♂
0.45	0.48	0.38	0.98	0.44	0.51	1.10	0.96	1.33	1.13	2.46	2.16
0.51	0.52	0.56	0.73	0.58	0.69	0.67	1.10	1.10	1.31	1.62	1.38
0.63	0.68	0.53	0.84	1.13	0.50	0.81	0.80	0.97	1.30	1.60	2.71
0.67	0.50	0.75	0.71	1.02	0.56	0.81	0.89	1.17	0.77	1.30	3.21
0.75	0.62	0.66	0.67	0.58	0.41	1.37	1.52	1.64	1.34	1.15	1.13
0.48	0.67	0.66	0.53	0.55	0.59	0.90	0.61	1.55	1.48	1.25	2.00
0.69	0.59	0.42	0.68	0.82	0.69	0.71	0.45	1.11	1.46	1.72	1.17
0.50	0.47	0.77	0.56	0.76	0.44	0.63	1.08	1.48	1.11	1.23	1.91
S♀	U♀	S♀	U♀	S♀	U♀	S♀	U♀	S♀	U♀	S♀	U♀
0.43	0.37	0.43	0.61	0.63	0.83	0.36	0.64	0.76	1.15	0.85	1.53
0.32	0.32	0.38	0.41	0.68	0.84	1.68	0.69	0.92	1.42	2.03	1.09
0.53	0.58	0.54	0.55	0.57	0.69	0.46	0.93	0.96	0.88	2.65	1.63
0.37	0.67	0.56	0.55	0.55	0.73	0.69	0.89	0.84	0.51	0.89	1.60
0.45	0.62	0.80	0.48	0.92	0.82	0.78	0.73	0.82	1.16	2.12	2.05
0.39	0.37	0.43	0.36	0.66	0.51	0.84	1.69	1.34	1.20	2.76	2.66
0.67	0.49	0.51	0.49	0.58	0.43	0.82	0.97	0.96	1.13	1.59	1.98
0.47	0.38	0.49	0.42	0.65	0.53	0.86	0.76	0.76	1.21	1.14	1.90

and the remaining half of the observations as those belonging to the unknown. This is true both for the data on the males and the females. Thus the dosage for both the so-called standard and unknown are the same. In a preliminary analysis it was found that when the data were plotted as a log dose-response relationship an exponential function was apparent. When reconsidered as a log dose-log response relationship a straight line function was evident. Therefore, this log dose-log response was used for further analysis of the data.

These data (Table II) were subjected to analysis by the statistical methods of Bliss (1944), Irwin (1937), and Bülbring (1935). Table III illustrates the predictable error when analyzed by the method of Irwin and Bülbring. By this method 3 dose levels of both the standard and unknown were employed. At each dose level 16 animals (8 males and 8 females) were employed for both the standard and the unknown. Thus under these conditions a total of 96 animals was employed. At the dosage levels of 5, 10, and 20 micrograms an error range of -45 to $+84$ per cent was found at $P = 0.95$. When the same number of groups of animals were employed at the range of 20 to 160 micrograms the error range was considerably reduced. At 20, 40, and 80 micrograms a range of -24 to $+32$ per cent was found while at the levels of 40,

TABLE III. RELATIVE POTENCIES AND ERRORS BY THE METHOD OF IRWIN
(Relationship between log dose and log response)

Number of chicks in each group		Concentration of testosterone propionate used μg.	Ratio of potency standard / Unknown	Errors $P=0.95$	
Males	Females			Low %	High %
8	8	5, 10, 20	100/90.6	45	84
8	8	20, 40, 80	100/101	24	32
4	4	20, 40, 80	100/117	43	73
8	8	40, 80, 160	100/118	17	21

80, and 160 micrograms the error range at $P=0.95$ was -17 to $+21$ per cent.

When a total of 48 animals was employed instead of 96 animals the error range increased considerably from -24 and $+32$ per cent to -43 and $+73$ per cent.

In Table IV the data are considered by the method of Bliss (1944). Here 2 dose levels of both the standard and the unknown were employed and the number of animals in each group is 16 (8 males and 8 females) in three comparisons and 12 (6 males and 6 females) in two comparisons.

TABLE IV. RELATIVE POTENCIES AND ERRORS BY THE METHOD OF BLISS
(Relationship between log dose and log response)

$$\frac{\text{High Dose of Standard}}{\text{Low Dose of Standard}} = \frac{\text{High Dose of Unknown}}{\text{Low Dose of Unknown}}$$

Number of chicks		Concentrations of testosterone propionate used	$M \pm S_m$	Error $P=0.95$	
Males	Females	μg.		Low %	High %
8	8	40, 160	128 ± 20.7	27	38
6	6	40, 160	129 ± 24.3	31	46
8	8	20, 80	87 ± 14.4	27	38
6	6	20, 80	95 ± 20.4	35	54
8	8	20, 40	102 ± 22.6	38	56

When 12 animals were employed per group a greater error of the potency ratio was found than when 16 animals were used but the difference was not particularly striking. At the levels of 40 and 160 micrograms groups of 16 (total animals was 64) an error range of −28 and +38 per cent was found as compared to an error range of −31 and +46 per cent for groups of 12 (total animals was 48). In a second instance where groups of 16 and 12 were compared at the 20 and 89 microgram levels, it was found that an error range of −27 to +38 per cent was found for the groups of 16 while the groups of 12 showed an error range of −35 to +54 per cent.

The highest error range of the potency ratio was found at the levels of 20 and 40 micrograms in spite of the fact that 16 animals were employed in each group. This range of error was from −38 to +56 per cent.

No significant difference was found in the slopes of the unknown and standard by either method of calculation.

DISCUSSION AND CONCLUSION

The data presented in this report confirm the findings of Klemper et al. (1942) as to the accuracy of the assay of androsterone. These workers reported a mean error of 13 per cent for 24 determinations of androsterone between 20 and 40 micrograms. For 16 determinations of androsterone between 20 and 40 micrograms we have found a mean error of 12 per cent. For 39 determinations between 10 and 50 micrograms these workers found a mean error of 24.6 per cent, while for the range 10 to 40 micrograms we have found a mean error of 24 per cent.

Still to be determined is the adaptability of this method to the assay of other androgens in terms of androsterone.

The method for the assay of testosterone propionate as presented in this paper appears to be of value both from the standpoint of sensitivity and reproducibility. Within the range of 20 to 160 micrograms the use of 48 animals on the unknown run in parallel with 48 animals on the standard accuracies of better than 32 per cent in potency ratio ($P=0.95$) can be realized. With the use of 32 animals on the standard and 32 chicks on the unknown run simultaneously, errors in potency ratio better than 38 per cent ($P=0.95$) can be realized between the dosage range of 20 to 160 micrograms. Recently Mathieson and Hays (1945) have demonstrated the feasibility of assaying testosterone propionate in castrate male rats using the experimental design of Bliss (1944). The expected error of the potency ratio using a total of 32 rats was similar to the error range found using 64 chicks.

SUMMARY

The utility of the Frank et al. (1942) chick comb method for the assay of androsterone has been confirmed. Using 16 to 20 chicks per

group and working in the range of 20 to 40 micrograms of androsterone a mean experimental error of 13 per cent was found.

A method is described for the assay of testosterone propionate in oil solution. The method employs the essential details described by Frank et al. (1942) except that the experimental design was changed so that the unknown was run in parallel with the standard. Within the range of 40 to 160 micrograms of testosterone propionate and with the use of a total of 96 chicks an accuracy of -17 to $+21$ per cent ($P = 0.95$) in the determination of the potency ratio was realized.

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STUDIES ON THE BIOASSAY OF HORMONES

THE RELATIVE REACTIVITY OF THE COMB OF VARIOUS BREEDS OF CHICKS TO ANDROGENS[1]

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IT HAS been known for some time that various breeds of fowls differ as to their practicability for use in androgen assays when the comb is used as the test organ. Thus the White and Brown Leghorns and the English Game Cock have been used on the basis of their relatively high sensitivity to androgens. The interest in the difference in comb reactivity goes beyond the question of utility of various breeds in the androgen assay. The reasons for the differences in the reactivity may serve as a valuable clue to the factors which operate in the reactivity of the comb to androgens as well as the mechanism of action of the androgens on the comb.

This communication deals with experiments on the reactivity of the combs in various species of fowls to androgens when the hormone was administered by direct application to the comb. This procedure should rule out the question of the endogenous material and the metabolism or inactivation of the hormone in the body. In these experiments we are dealing only with the direct stimulation and the local inactivation of the hormone at the site of the comb.

ANIMALS, MATERIALS, METHODS

The three breeds of chicks, the White Leghorn, the Rhode Island Red, and the Barred Rock, were obtained from the Kerr Chickeries, Frenchtown, N. J., and were received in Cleveland one to two days after hatching. The animals were kept in thermostatically controlled brooders and fed chick starting mash and water exclusively. The chicks were 2 to 3 days of age at the start of the experiment. The total dose of testosterone propionate[2] was contained in 0.35 cc. of corn

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[1] Supported in part by a grant from the National Research Council Committee on Endocrinology.

[2] The testosterone propionate was kindly supplied by Ciba Pharmaceutical Products, Inc.

oil and administered once daily for seven days. Five-hundredths of a cc. of the hormone solution was dropped on the comb from a 1 cc. tuberculin syringe fritted with a number 24 hypodermic needle. Twenty-four hours after the last hormone application, the animals were sacrificed and body weight and comb weights were determined. The animals were autopsied at 9 to 10 days of age. The comb responses are expressed as the ratio of the comb weight in milligrams to the body weight in grams.

Ten logarithmic dosage intervals of testosterone propionate were studied for two breeds of chicks and 14 intervals for the White Leghorns. The dosage range was from 40 μg. to 20.48 mg. per animal total dose in all three breeds.

EXPERIMENTAL

The body weight of the three breeds studied did not vary significantly. The mean body weights for the male White Leghorns, Rhode Island Reds, and Barred Rocks were 62, 61 and 58 grams, respectively, while the mean body weights of the females of the same species were 58, 59 and 57, respectively. The number of animals per group varied from 34 to 49 animals.

Table I presents a summary of the comb responses to varying concentrations of testosterone propionate. The comb size is represented as the ratio of comb weight in milligrams to the body weight in grams. The data have been considered from a number of points of view which may be listed as follows:

(A) Analysis of control ratios of the respective breeds.
(B) Comparative amounts of androgen necessary to produce a statistically significant increase in comb ratio.
(C) Determination of respective slopes for each of the three breeds such that these slopes are not significantly different among themselves. From these data calculate relative amounts of testosterone propionate for various breeds necessary to produce similar rate of increment in comb ratio with increasing dose.
(D) Comparison of maximum slopes for the three breeds.
(E) Comparison of maximum comb ratios attainable by three breeds after testosterone propionate stimulation.

(A) In none of the three breeds do we find a significant difference between the comb sizes of male and female chicks. On the other hand, there is a distinct difference between the sizes of the combs of the White Leghorn and Rhode Island Red chicks. This difference in comb ratio was found for both males and females. Thus, the comb ratio of White Leghorn males was 0.38 ± 0.01 as compared to 0.25 ± 0.01 for the Rhode Island Reds. The female White Leghorn chicks had a comb ratio of 0.36 ± 0.01 while the Rhode Island Reds of the same

sex had a value of 0.23 ± 0.01. An even greater difference has been found between the comb ratios of the White Leghorn and Barred Rock chicks for both the males and females.

TABLE I. THE RELATIVE REACTIVITY OF THE COMBS OF WHITE LEGHORNS, RHODE ISLAND RED, AND BARRED ROCK CHICKS TO TESTOSTERONE PROPIONATE WHEN ADMINISTERED BY DIRECT APPLICATION TO THE COMB

Breed of chick	Amount administered μg.	Number of chicks		Ratio = Comb in mg. / B.W. in g. ± S.E.	
		M	F	M	F
White Leghorn	0	62	56	0.38 ± 0.01	0.36 ± 0.01
	2	13	6	0.46 ± 0.04	0.43 ± 0.06
	5	28	34	0.55 ± 0.02	0.47 ± 0.02
	10	32	31	0.63 ± 0.03	0.52 ± 0.02
	20	18	45	0.65 ± 0.04	0.67 ± 0.02
	40	36	51	0.77 ± 0.05	0.69 ± 0.02
	80	46	38	1.03 ± 0.05	0.91 ± 0.05
	160	29	36	1.53 ± 0.10	0.54 ± 0.09
	2,560	25	13	1.74 ± 0.07	1.74 ± 0.15
	5,120	12	12	1.88 ± 0.15	1.98 ± 0.15
	20,480	8	13	1.89 ± 0.22	1.82 ± 0.16
Rhode Island Red	0	47	29	0.25 ± 0.01	0.23 ± 0.01
	40	14	11	0.33 ± 0.01	0.33 ± 0.02
	80	13	11	0.36 ± 0.03	0.40 ± 0.04
	160	9	14	0.48 ± 0.05	0.49 ± 0.05
	640	9	16	0.85 ± 0.10	0.82 ± 0.10
	1,280	15	10	0.91 ± 0.07	0.87 ± 0.09
	2,560	32	21	0.92 ± 0.05	1.03 ± 0.07
	5,120	11	12	1.14 ± 0.07	1.03 ± 0.08
	20,480	—	19	—	1.10 ± 0.08
Barred Rock	0	36	37	0.21 ± 0.01	0.20 ± 0.01
	40	13	10	0.25 ± 0.02	0.22 ± 0.02
	80	12	13	0.23 ± 0.02	0.29 ± 0.02
	160	9	14	0.36 ± 0.04	0.32 ± 0.02
	640	12	17	0.54 ± 0.03	0.55 ± 0.03
	1,280	11	15	0.61 ± 0.02	0.63 ± 0.04
	2,560	24	31	0.85 ± 0.01	0.82 ± 0.04
	5,120	9	15	0.97 ± 0.09	1.07 ± 0.08
	20,480	12	10	1.01 ± 0.11	1.02 ± 0.07

(B) Males of the breeds White Leghorn, Rhode Island Red and Barred Rock, respectively, require 2, 30 and 40 μg. of the steroid for a significant comb stimulation (20 per cent increase). Compared with the White Leghorn male, we may say that the Rhode Island Red male requires 15 times as much hormone, while the Barred Rock required 20 times as much hormone. Similar consideration of the females indicates that the Rhode Island Red requires 10 times as much hormone as the White Leghorn female and that the Barred Rock requires 20 times more testosterone propionate.

It must be remembered that the actual increment in comb ratios are largest for the White Leghorn and smallest for the Barred Rock chicks. Thus at the level of response considered the actual incre-

ments in comb weight represented are 0.07 mg. per gram of body weight for the White Leghorn, 0.05 mg. per gram for the Rhode Island Reds and 0.04 mg. per gram for the Barred Rocks.

(C) The reactivity of three breeds of chicks, as judged by the amount of hormone needed to attain a certain arbitrary slope of response may be compared. The choice of the slopes was made on the basis of the maximum slopes attainable by the Rhode Island Red and Barred Rock chicks. A portion of the response curve of the White Leghorn chicks was then taken which had a slope similar to those of the less reactive breeds. If the slopes are not significantly different then it is possible to get a measure of the relative comb reactivity of the various breeds by measuring the displacement of the various curves.

TABLE II. THE MAXIMUM SLOPES OF COMB RESPONSE ATTAINABLE BY RHODE ISLAND RED AND BARRED ROCK CHICKS
THE WHITE LEGHORN COMB RESPONSE CURVE WAS TAKEN WHERE THE SLOPE WAS NOT SIGNIFICANTLY DIFFERENT FROM THAT OF THE OTHER BREEDS
(LOGARITHM DOSE-RESPONSE)

Breed of chicks	Sex	Dosage levels of testosterone propionate μg.	Total number of animals	Slope $b \pm$S.E.
White Leghorn	M	20; 40; 40	100	0.677 ± 0.107
	F	10; 20; 40; 80	165	0.357 ± 0.023
Rhode Island Red	M	80; 160; 640	31	0.545 ± 0.026
	F	80; 160; 640	41	0.485 ± 0.110
Barred Rock	M	640; 1280; 2560	47	0.537 ± 0.101
	F	640; 1280; 2560; 5120	78	0.484 ± 0.110

Table II illustrates the various dosage levels that were employed to get, first, the maximum slopes for the Rhode Island Red and Barred Rock chicks and, secondly, a range of dosages for the White Leghorn chicks which yielded slopes not significantly different than those of the other two breeds. All considerations were log dose-response relationships. The statistical analysis was done for both male and female chicks.

For the male chicks the slopes considered were 0.677 ± 0.107, 0.545 ± 0.026, and 0.537 ± 0.101 for the White Leghorn, Rhode Island Red, and Barred Rock chicks respectively. For the female chicks the slopes were 0.357 ± 0.023, 0.485 ± 0.110, and 0.484 ± 0.11 for the White Leghorn, Rhode Island Red and Barred Rock chicks respectively.

In Table III the significance of difference between the slopes was tested by the method of Fischer (1934). The slopes of both the Rhode Island Red and Barred Rock chicks were compared to the slope of the

White Leghorn. In no instance for either the response of the male or female chick's combs was it possible to demonstrate a significant difference in the slopes.

The sensitivities of the respective breeds may be represented as percentages of that of the White Leghorn chicks, the latter sensitivity being considered as 100 per cent. The calculations were carried out for both the males and females of each breed by the method of Bülbring (1935). Considering the male White Leghorn chick's comb to have a sensitivity of 100 per cent, the Rhode Island Red chick's comb was found to have a sensitivity of 10 per cent and the combs of

TABLE III. SIGNIFICANCE OF DIFFERENCE BETWEEN SLOPES LISTED IN TABLE II

Breeds of chicks compared	Sex	Fisher's t	Number of chicks	P
White Leghorn and Rhode Island Red	M	0.898	129	0.3–0.4
	F	0.955	194	0.3–0.4
White Leghorn and Barred Rock	M	0.868	145	0.3–0.4
	F	0.981	241	0.3–0.4

the male Barred Rock a sensitivity of only 1.8 per cent. The comparative comb sensitivity of the females was similar to that found for the males. When the female White Leghorn comb was assigned a sensitivity of 100 per cent the Rhode Island Red chick's comb was found to be 8.9 per cent and the Barred Rock chick's comb was found to be 1.8 per cent. The error of the determinations at $P=0.95$ was quite low. The maximum error range being -33 to $+48$ per cent and the minimum error range being -19 to $+23$ per cent.

(D) In the preceding analysis it was found that the White Leghorn chick required far less testosterone propionate to attain a slope similar to that of the other two breeds of chicks. Further the slopes for the Rhode Island Red and Barred Rock chicks were the maximal slopes attainable considering at least three dosage levels. However, the White Leghorn logarithm dose-response curve was not the maximum attainable. In Table IV this point is illustrated for both male and female chicks. Since the maximum slopes for the response relationships of the Rhode Island Red and Barred Rock did not differ significantly, only the maximum slope of the latter group was used for comparison with the White Leghorns. The maximum slope for the male White Leghorn logarithm dose-response relationship was 1.215 ± 0.147 as compared to 0.537 ± 0.101 which was found for the maximum slope of the Barred Rock males. The difference in slopes was significant as seen by the Fischer's t value of 3.027 which indicates a value of P below 0.01. The difference in maximum slopes for female logarithm dose-response curve was also significant. The

White Leghorn females had a maximum slope of 1.361 ± 0.100 as compared to a slope of 0.484 ± 0.110 for the Barred Rock females. The t value was 8.240 indicating a P value well below 0.01.

TABLE IV. COMPARISON OF MAXIMUM SLOPES ATTAINED BY REGRESSION OF WHITE LEGHORN AND BARRED ROCK COMBS ON TESTOSTERONE PROPIONATE. (RHODE ISLAND RED MAXIMUM SLOPES NOT SIGNIFICANTLY DIFFERENT FROM THOSE OF BARRED ROCK)

Sex	Maximum White Leghorn slope[1] ± S.E.	Maximum Barred Rock slope ± S.E.	Total Number of chicks	t	P
M	1.215 ± 0.147	0.537 ± 0.101	158	3.027	0.01
F	1.361 ± 0.100	0.484 ± 0.110	223	8.240	0.01

[1] Derived from 40, 80, and 160 μg. doses of testosterone propionate.

(E) In Table V another means of evaluating the reactivity of the comb is considered, namely, the maximum size attainable by the combs of the respective breeds at any dose. When considered on the basis of comb size alone or on the magnitude of the comb ratio the White Leghorn breed outranks the other two breeds. But it must be remembered that the White Leghorn chicks start with larger combs.

TABLE V. COMPARATIVE MAXIMUM COMB RATIOS ATTAINED BY THREE BREEDS OF CHICKS AFTER APPLICATION OF TESTOSTERONE PROPIONATE

Breed of chick	Males		Females		Mean increase male and female 0/0
	Control ratio	Maximum increase 0/0	Control ratio	Maximum increase 0/0	
White Leghorn	0.38	396	0.36	415	405
Rhode Island Red	0.25	357	0.23	364	361
Barred Rock	0.21	344	0.20	426	385

If one considers the data on the basis of percentage increment in comb ratio over and above that of the oil treated controls no significant difference among the groups was found. These calculations are presented in Table V. In the male chicks the maximum increment in comb ratios was 395, 357, and 344 per cent respectively for the White Leghorns, Rhode Island Reds, and Barred Rocks. For the female chicks the maximum increase was 415, 364, and 426 per cent respectively for the various breeds in the same order.

DISCUSSION

The data presented in this report define in a quantitative manner the difference in reactivity of combs of various breeds of chicks to

androgens. The White Leghorn comb is by far the most reactive to androgens when evaluated by any of three criteria: (1) the minimal amount of hormone necessary to produce a statistically significant increment in comb weight; (2) the amounts of hormone necessary to produce a certain arbitrary slope of response on a log dose-response basis; and (3) the maximum attainable slope of response again calculated as a log dose-response relationship.

Since the hormone was administered by direct application the difference in reactivity must be considered as a function of the comb only. That is, no peculiar hormone metabolic differences in the organism as such could be responsible for the difference in the comb reactivity. At least the same quantity of the same hormone was available to the comb.

What then are the possible factors for the differences in the comb reactivities among breeds? First, a difference may exist with respect to the permeability of the comb to hormone. Second, an enzyme system may be present in greater abundance in the poorer reactors which tend to inactivate the administered hormone more efficiently. Third, the abundance of enzyme systems which are concerned with comb tissue synthesis and organization may vary significantly. The responsibility for the differences in reactivity may not be a single factor but a combination of factors.

Further work is in order, particularly on the possible quantitative differences in comb enzyme systems to elucidate this problem.

SUMMARY

The quantitative differences in the reactivity of the combs of three breeds of chicks has been studied. The androgen testosterone propionate was applied to the combs in oil. The dosage range investigated was from 2 micrograms to 20,480 micrograms for the White Leghorn male and female chicks and 40 micrograms to 20,480 micrograms for the Rhode Island Red and Barred Rock breeds.

No significant difference was found in the comb ratios of corn oil control of male and female chicks for any of the three breeds studied but a significant difference was found for the control ratios of the three breeds studied. White Leghorn chicks showed the largest combs, the Barred Rocks the smallest, and the combs of the Rhode Island Reds were intermediate. This order of comb size of control chicks was true for both the males and females.

On the basis of the minimal quantity of testosterone propionate to produce a 20 per cent increment in comb ratio the male White Leghorns were 15 times as sensitive as the Rhode Island Reds and 20 times as sensitive as the Barred Rocks. Similarly the female White Leghorn combs were 10 times as sensitive as those of the Rhode Island Reds, and 20 times those of the Barred Rocks.

The comparative sensitivities of the combs of the three breeds

was evaluated by picking portions of the log dose-response curves where the slopes of all three breeds were not significantly different and using the displacement of the curves as another measure of the relative sensitivity of the various breeds. Under these conditions and expressing the sensitivity of the male White Leghorn chick comb as 100 per cent the sensitivity of the Rhode Island Red was found to be 10 per cent and that of the Barred Rock as 1.8 per cent. In the female chicks the relative comb sensitivities was similarly White Leghorn, 100 per cent; Rhode Island Red, 8.9 per cent; and Barred Rock, 1.8 per cent.

A third criterion of sensitivity of the respective combs to androgen was maximum slope attainable using a log dose-response relationship for at least three points. No significant difference in maximum slope was found for the Rhode Island Red and Barred Rock chick combs but the White Leghorns showed a significantly greater slope.

Finally, an evaluation of the maximum percentage increase in comb size by androgen stimulation revealed that no significant difference could be demonstrated among the three breeds for either male or female chicks.

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THE METABOLISM OF SOME CHEMICAL DEGRADATION PRODUCTS OF ESTROGENS: WESTERFELD'S LACTONE, BIS-DEHYDRO-DOISYNOLIC ACID, ESTROLOLACTONE AND β-ESTRADIOL

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THERE IS a large body of evidence that natural estrogens are inactivated in the liver. *In vitro* and *in vivo* studies are in agreement on this. However, there is a remarkable paucity of evidence as to what actually happens to the administered estrogen. A small portion is converted into other estrogens and excreted in the urine (Doisy, et al., 1942; Doisy, 1942; Pincus, 1946). The recovery of estrogenic activity is increased if the urine is boiled with zinc and hydrochloric acid but there still remains a large proportion of the estrogen for which we are unable to account at present (Smith, 1941).

Chemical degradation of estrogens has yielded estrogenically active materials. It was decided to study some of these which were available in an attempt to shed light upon the problem.

MATERIAL AND METHODS

The sodium salt and the 3-methyl ether of bis-dehydro-doisynolic acid were supplied by Dr. C. R. Scholz of Ciba Pharmaceutical Products, Incorporated (Figure 1).

The Westerfeld's lactone acetate was prepared by the peroxide oxidation method of Mather from estrone acetate supplied by Dr. E. L. Foreman of Lakeside Laboratories and by Dr. Scholz. A small sample was also obtained from Dr. Gregory Pincus. Both our preparation and Dr. Pincus' had an uncorrected melting point of 148°–150°C and a mixture of the two had the same melting point.

The β-estradiol was prepared for us by Dr. Martin Hoffman, of McGill University, from crude material supplied by Dr. Erwin Schwenk of the Schering Corporation. The preparation employed in these studies was of high purity and had a melting point of 220°–221°C.

The estrololactone acetate, the structure of which has not been elucidated as yet, was supplied through the courtesy of Drs. Pincus and R. P. Jacobson of the Worcester Foundation for Experimental Biology.

The same methods were employed as in our previous studies on estrogen metabolism (Segaloff, 1943). Young adult female rats, purchased from Maguran Farms, were spayed. They were then primed with 50 μg. of estrone

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in 0.1 cc. of peanut oil, injected subcutaneously. The test injection was then given in 0.05 cc. of 5% benzyl alcohol in sesame oil either subcutaneously or intrasplenically with the spleen *in situ*. In an attempt to get positive results with Westerfeld's lactone acetate and β-estradiol, we injected animals with 0.1 cc. of a saturated solution intrasplenically with the spleen *in*

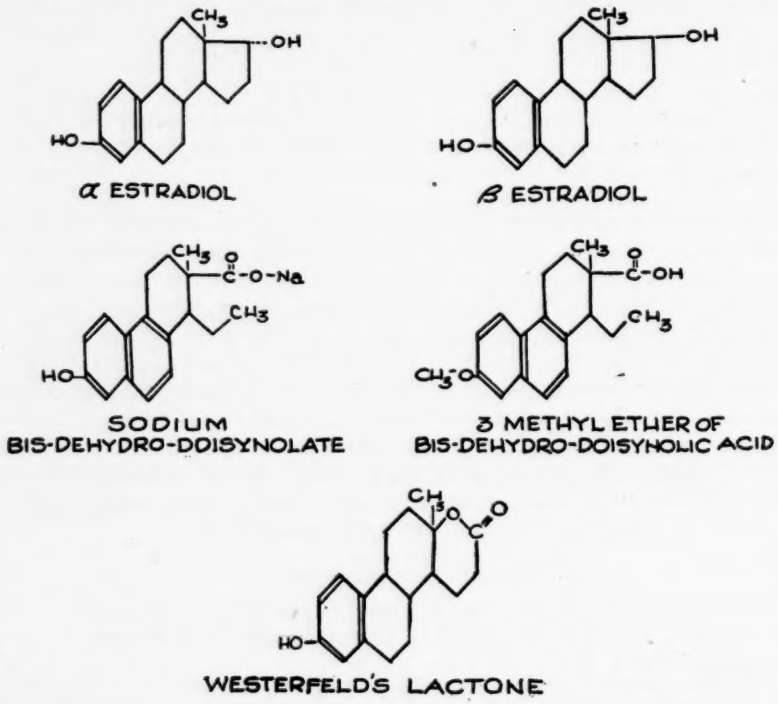

FIG. 1

situ. The 0.1 cc. contained 500 μg.; 0.1 cc. of the saturated solution of estrololactone acetate was also injected intrasplenically; this volume contained 1 mg. Vaginal smears were taken with a moistened cotton-tipped toothpick, 48, 54, 60 and 72 hours after the injection. The smears were air-dried and stained with hematoxylin and eosin. A smear which showed only cornified epithelial cells at one or more of these times was classified as representing a positive vaginal estrus response for that animal. At least 20 animals, but not more than 40, were employed at each of the levels being reported.

RESULTS

The results are presented in Table 1. The methyl ether of bis-dehydro-doisynolic acid and its sodium salt are considerably more active by subcutaneous injection than is α-estradiol. It is apparent

that Westerfield's lactone acetate is much less active by subcutaneous injection than is the sodium salt or the methyl ether of bis-dehydro-doisynolic acid. β-Estradiol has about the same activity by subcutaneous injection as Westerfeld's lactone acetate and the estrololactone acetate had far less activity by subcutaneous administration than any of the other compounds. In addition, Westerfeld's lactone acetate is inactivated in the liver to a great extent since 500 μg. intrasplenically

TABLE I. THE ESTROGENIC ACTIVITY OF NATURAL ESTROGENS AND DEGRADATION PRODUCTS THEREOF WHEN ADMINISTERED BY DIFFERENT ROUTES

	Subcutaneous injection		Intrasplenic injection		Ratio
	Dose—	Estrus	Dose—	Estrus	S.C.:I.S.
	μg.	%	μg.	%	
α-Estradiol	1.5	30	50	0	
	1.75	50	75	50	1:43
	3.0	85	150	90	
β-Estradiol	25	33	500*	20	
	40	50			
	75	72			
Westerfeld's lactone acetate. (From Drs. Pincus & Jacobson)	25	20			
	37.5	50			
	50	70			
Westerfeld's lactone acetate. (My preparation)	20	20	500*	10	
	30	50			
	37.5	67			
Estrololactone acetate	100	6	1000*	19	
	325	50			
	500	83			
3-methyl ether of bis-dehydro-doisynolic acid	0.20	10	0.10	0	
	0.35	55	0.20	50	1:0.57
	0.50	91	0.30	81	
Sodium-bis-dehydro-doisynolate	0.5	10	0.26	30	
	0.75	45	0.375	50	1:0.5
	1.0	90	0.5	85	

* 0.1 cc. of a saturated solution.

with the spleen *in situ* elicited only a 10% estrus response. This is thirteen times the amount required for 50% estrus by subcutaneous injection, so that the ratio of subcutaneous to intrasplenic is greater than thirteen. Our preparation and that supplied by Dr. Pincus showed essentially the same activity by subcutaneous injection. β-Estradiol is also inactivated in the liver since thirteen times the 50% estrus dose by subcutaneous injection only produced a 20% estrus response. Therefore, the ratio for β-estradiol is also greater than thirteen.

Both sodium bis-dehydro-doisynolate and the 3-methyl-ether of bis-dehydro-doisynolic acid are more active when given intrasplenically than when given subcutaneously (Table 1). The subcutaneous-

intrasplenic ratios for the 50% estrus levels are 1:0.57 and 1:0.5 respectively.

DISCUSSION

Westerfeld's lactone acetate seems to be metabolized in a manner similar to its parent compound, estrone, which was previously shown to be inactivated by the liver. In the studies on estrone the same technique was employed (Segaloff, 1943). This compound has aroused much interest since its preparation. Smith and Smith (1944) have demonstrated that despite its low estrogenic potency, it is more potent than estrone in its ability to increase the output of gonad stimulating hormone from the pituitary of the rat. The Smiths (1946) believe that Westerfeld's lactone, or some similar degradation product, plays a key role in the initiation of menstruation in man.

Estrololactone acetate also appears to be inactivated by the liver. Its low estrogenic activity, by a uterine weight assay method, as well as by vaginal smears, has been reported previously by Pincus (1947). He also reports that it is as potent as estrone in the inhibition of pituitary gonadotrophin, but unlike estrone it does not produce the release of pituitary corticotrophin.

β-Estradiol is also inactivated by the liver, but again its low potency precludes an exact estimation of the degree of inactivation.

The results with the methyl ether and sodium salt of bis-dehydro-doisynolic acid were surprising in view of the preceding discussion. It appears that the rat's liver is capable of activating or increasing the estrogenic potency of these compounds. We previously reported (1944) such *in vivo* activation of synthetic proestrogens.

It appears to be of great interest that rupture of the 5 membered ring in estrone can lead to compounds of such varying estrogenic potency which are handled by the liver in totally different ways.

SUMMARY

α-Estradiol and some estrogen degradation products were injected subcutaneously. It was observed that they could be arranged in the following order of decreasing estrogenic potency: 3-methyl ether of bis-dehydro-doisynolic acid > sodium bis-dehydro-doisynolate > α-estradiol > Westerfeld's lactone acetate > β-Estradiol > estrololactone acetate.

When α-estradiol was injected intrasplenically instead of subcutaneously, 43 times as much was required to produce vaginal estrus in one half of the animals. Intrasplenic injections of β-estradiol, Westerfeld's lactone acetate and estrololactone acetate with 12, 17 and 3 times the subcutaneous 50% estrus dose respectively failed to produce estrus in 50% of the animals. Intrasplenic injections of the 3-methyl ether of bis-dehydro-doisynolic acid and sodium bis-dehydro-doisynolate, on the other hand, required only one half as much to produce vaginal

estrus in 50% of the animals as when the estrogen was administered subcutaneously

These results are interpreted as indicating that the liver inactivates α and β-estradiol, Westerfeld's lactone acetate and estrololactone acetate but enhances the estrogenic potency (activates) the 3-methyl ether of bis-dehydro-doisynolic acid and sodium bis-dehydro-doisynolate.

ACKNOWLEDGMENTS

We would like to express our appreciation to the men mentioned in the text for their generous supplies of material.

We would like particularly to thank Dr. Alan Mather for making his unpublished data and methods available to us.

ADDENDUM

Since this paper was submitted for publication a paper by Jacobson has appeared in which the preparation of estrololactone is discussed in detail. Such chemical data as are available are also discussed. (Jacobson, R. P. *J. Biol. Chem.* 171: 61. 1947.)

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VISCOSIMETRIC DETERMINATION OF THE HYALURONIDASE CONTENT OF SPERMATOZOA

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WITH THE observation that the dispersion of the cumulus cells surrounding the rat ovum was necessary to enable fertilization of the egg by sperm, and that this dispersion could be brought about by a heat labile substance produced by spermatozoa, Pincus & Enzmann (1935), the relationship of these substances to certain clinical aspects of sterility has received increasing attention.

Factors with similar diffusing properties have been obtained from numerous sources including culture filtrates, invasive bacteria, a large variety of organs and tissues, neoplasms included, snake venom and leeches. Chain and Duthie (1940) noted a marked mucolytic activity of these extracts, characterized by a rapid fall in the viscosity of synovial fluid and the liberation of reducing substances. Further investigation by others revealed that these spreading factors are closely associated, if not identical, with a group of enzymes that hydrolyze hyaluronic acid, and are known as hyaluronidases.

Rowlands (1944) demonstrated that with the addition of an enzyme from a semen filtrate, the number of spermatozoa required to accomplish artificial insemination in rabbits, was reduced one sixth. This work again suggested that the large number of spermatozoa was required for the fertilization of the mammalian ovum, in order to provide a concentration of enzyme sufficient to cause dispersion of the cumulus cells surrounding the ovum, so that one sperm could effectively fertilize the egg. The application of this concept to the study and treatment of human sterility (Eichenberger, 1946; Leonard and Kurzrok, 1945; Greenberg and Gargill, 1946; Swyer, 1946) has resulted in the need for a simple accurate method for the measurement of hyaluronidase content in fertile and infertile human semen.

The viscosimetric measurement of the degradation of hyaluronic acid by hyaluronidase in a system controlled with reference to pH and salt concentration, is probably the most reliable (McClean, 1941). For this reason, we undertook to adapt such a technique for

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application in the study of the hyaluronidase content of semen. Our purpose was to maintain the level of accuracy and reduce to a minimum involved procedures and mathematical computations, so that the test could have clinical application. In addition to its accuracy, the technique below has the further advantage of requiring only small volumes of substrate and semen.

The hyaluronic acid solution is a 0.25% of purified material in distilled water. The hyaluronic acid is prepared from umbilical cords.[1] This solution has a viscosity of approximately four times that of water, when checked with the Ostwald viscosimeter. The solution is quite stable and will keep in the refrigerator for two to three months, with only a barely perceptible diminution in viscosity. The substrate is prepared by mixing four volumes of 0.25% hyaluronic acid solution with one volume of a Molar Sodium Citrate buffer pH 4.6. This buffer is prepared by mixing one volume of Molar Citric Acid with two volumes of Molar Sodium Hydroxide.

The viscosity of the substrate solution is determined with an Ostwald viscosimeter (approximately 5 ml. capacity). The substrate, composed of 2.5 cc. of 0.25% hyaluronic acid to which 0.625 cc. of Molar Sodium Citrate buffer pH 4.6 has been added, is mixed with 1 cc. of 0.1 Molar Sodium Chloride, and the entire mixture is poured into the viscosimeter bulb. All readings are done in a bath at 37.5° C. Several flow times are recorded and the average of these is the viscosity of the substrate solution. The value may be considered constant for the life of the hyaluronic acid solution (2–3 months).

Determination of the viscosity reducing effect of semen:

1. The specimen is collected in a chemically clean container which has been sterilized. Collected in this fashion, the specimen can be kept in a refrigerator for as long as two weeks with no apparent change in its viscosity reducing ability. Figure 1 demonstrates flow curves done on the same specimen two weeks apart. For the determination, the semen specimen is diluted with an equal volume of 0.1 Molar Sodium Chloride and centrifuged until the supernatent is clear of any particles.

2. One cc. of the supernatent fluid and the substrate solution described above, are warmed separately to 37.5° C. for ten minutes in the bath.

3. The preparation from the semen is added to the substrate, and at this instant stop watch #1 is started. (This watch runs throughout the determination and gives the total reaction time.) The solution is shaken vigorously.

4. Using a small glass funnel, the mixture is carefully poured into the lower bulb of the viscosimeter. The mixture is drawn up into the

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[1] We are very grateful to the Schering Corporation, Bloomfield, N. J. for supplying us with the purified hyaluronic acid.

tube and when the meniscus reaches the upper mark on the tube, stop watch #2 is started. The time it takes for the mixture to fall from the upper to the lower mark is referred to as the flow time, and is recorded by stop watch #2. At the end of each run, readings are taken on both watches and the flow time (reading on watch #2 for each run), is recorded against the total reaction time-reading on watch #1 which runs continually from the start to the finish of the determination.

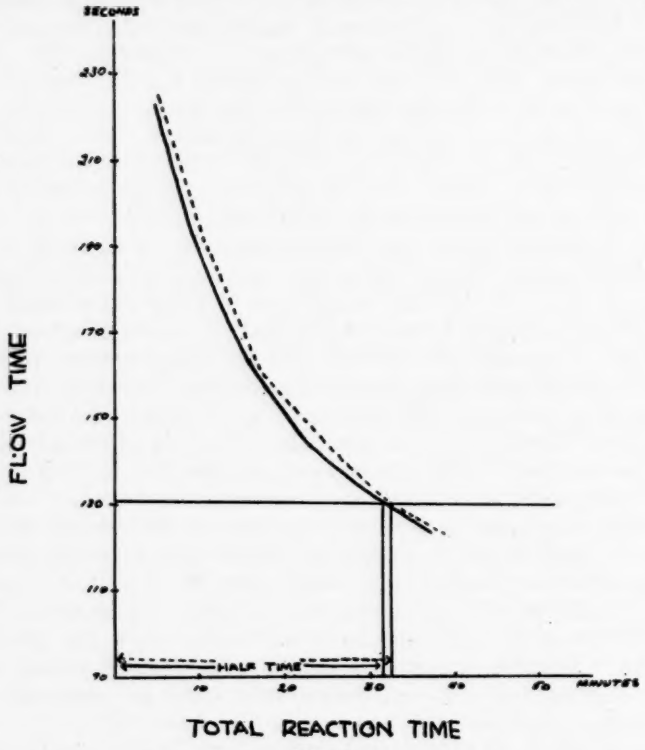

FIG. 1

Count 18,600,000 sperm/cc.
——— Feb. 1, 1947
-------- Same specimen Feb. 14, 1947.

The mixture is run at frequent intervals until the flow time of the enzyme system is less than one half that of the substrate. The end point is the total reaction time required to reduce the viscosity of the substrate to one half its original value.

Forty-four semen specimens were tested for enzymatic activity with this technique. The findings are graphically demonstrated in Fig. 2, in which the concentration of spermatozoa per cc. is plotted against the total reaction time required to reduce the viscosity of the

substrate to one half of its original value for each specimen. From this it is evident that but for three exceptions this time is inversely proportional to the concentration of spermatozoa per cc. Aspermic specimens failed to reduce the viscosity of the substrate. Figure 3 demon-

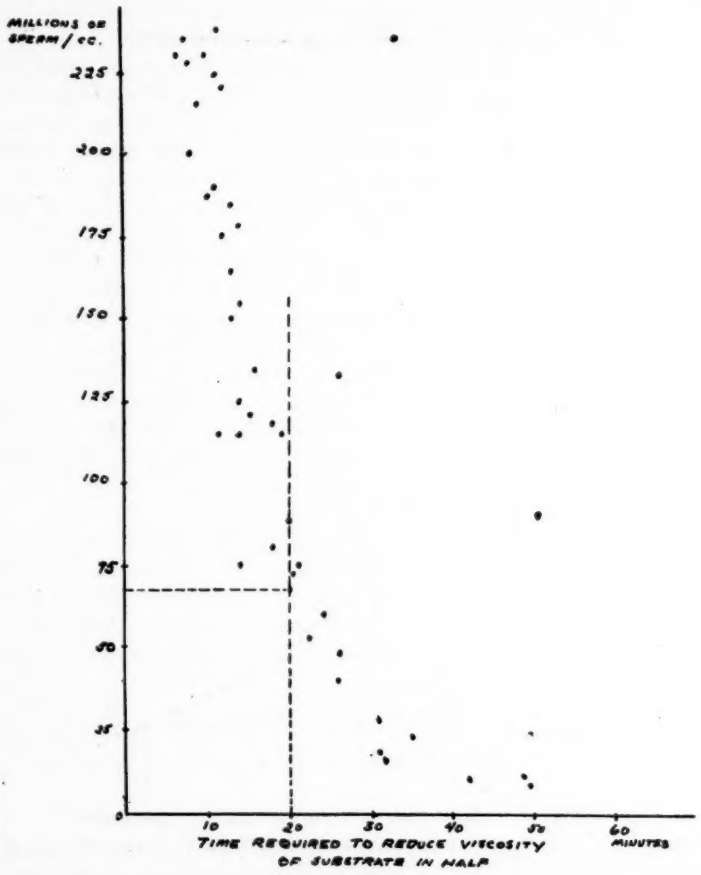

FIG. 2

strates characteristic flow curves for typical specimens. It is to be noted that all specimens, with the exception of the three noted above, containing more than 60,000,000 spermatozoa per cc. reduced the viscosity of the substrate to half value in 20 minutes or less.

Examination of the three specimens whose enzyme content could not be correlated to the sperm count, revealed some reason for the inconsistency in two of the specimens. One of these specimens, containing 215,000,000 spermatozoa was markedly viscus when received.

The viscosity six hours after it had been obtained by the patient was grossly much greater than normal. All other factors, volume, motility and morphology were within normal range. No evidence of prostatic infection was apparent in the specimen. This specimen, although with an apparent enzyme concentration characteristic of specimens con-

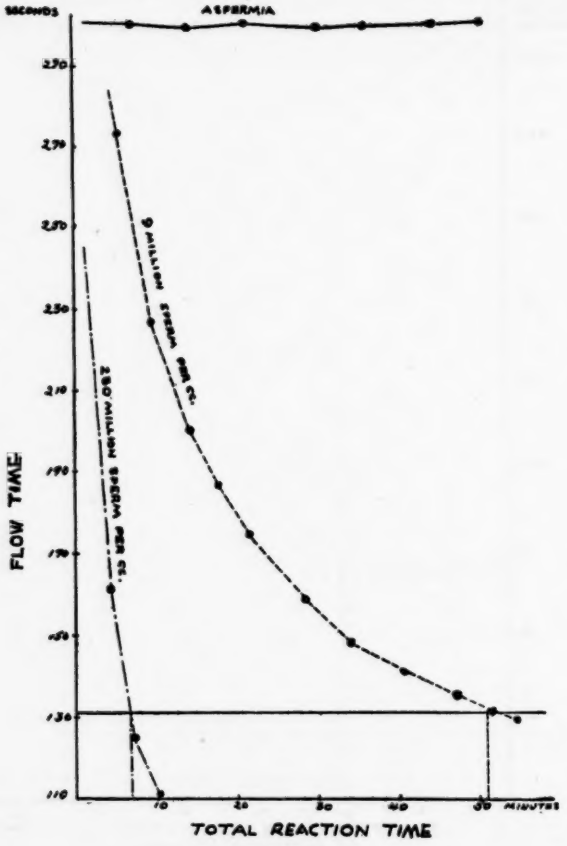

FIG. 3

taining less than 25,000,000 spermatozoa per cc. was from an individual who had manifest proof of his fertility. Repeat studies since then have yielded approximately the same results.

The second specimen contained 132,000,000 spermatozoa per cc. Volume, morphology and motility were within the normal range. Here too the viscosity was grossly much greater than normal. However in this instance there was definite evidence of a prostatic infection. Unfortunately we have been unable to maintain a follow-up on this patient.

The third specimen in this group contained 90,000,000 spermatozoa per cc. and was apparently normal in every other respect, volume, viscosity, motility and morphology. The concentration of the enzyme in this specimen is equivalent to that usually found in semen containing less than 15,000,000 spermatozoa per cc. Nothing is known concerning the fertility of this individual.

The concentration of hyaluronidase is apparently unrelated to morphology or motility. The apparent deficiency of the enzyme in very viscous specimens in spite of normal spermatozoa concentrations, indicates the need for more study in such instances. Fortunately one of these patients is available, and studies on the acid-phosphatase concentration of these specimens in conjunction with hyaluronidase estimations are being carried out at present. These findings will be reported at a future date.

Studies with aspermic specimens which failed to lower the viscosity of the substrate solution, reveal that these specimens have, however, the ability to diffuse in the skin when compared with a buffered control. These findings corroborate those of McClean (1941), that diffusion in the skin is caused by preparations that are too weak to be titrated viscosimetrically. Whether this diffusion is secondary to the presence of small amounts of hyaluronidase in the semen, or to other spreading factors, remains to be determined.

SUMMARY

A simple viscosimetric test for the determination of hyaluronidase in semen is described. The concentration of the enzyme as determined by this technique is proportional to the concentration of spermatozoa, with few exceptions. In two instances, abnormally viscous specimens failed to contain enzyme proportional to their sperm concentration. One semen specimen, apparently normal in all other respects, was also in this category. Aspermic specimens which could not be titrated viscosimetrically, demonstrated evidence of "spreading factor" when injected intracutaneously in the skin of rabbits.

ACKNOWLEDGMENTS

We are indebted to Dr. Perry Katzen for his cooperation in obtaining semen specimens used in this study.

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SOME FACTORS INFLUENCING THE LIBERATION OF HYALURONIDASE FROM TESTES HOMOGENATE AND SPERMATOZOA IN THE RAT[1]

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RECENT experiments concerning the effects of the enzyme haluronidase from sperm on mammalian ova have led to theoretical considerations of its role in reproduction (for discussion see Rowlands, 1944; Leonard, Perlman and Kurzrok, 1946). One of the problems has centered about the source of the enzyme obtained in the seminal plasma or extracts of testicular tissue, i.e., was it "secreted" by living sperm or germinal cells into the suspending medium, or was the enzyme liberation the reflection of a moribund cell population? The results of such studies would bear directly on any interpretation of semen assays as an index of fertility. It is evident from the experimental data to be presented that an increase in cell destruction is accompanied by an increase in the measurable hyaluronidase.

Methods

The methods of preparation of the testis homogenate and hyaluronidase determination were the same as previously described (Leonard et al., 1946). The enzyme unit is the turbidity reducing unit (TRU). It was possible to make a complete assay on a single testis and in many cases experiments were performed on one rat testis, the other serving as the control. Spermatozoa were taken from both the ductus deferens and the cauda epididymis of adult rats and were suspended in either Ringer's or 0.1 M acetate buffer (pH 6.0). Assays were made only on the cell-free supernatant fluid. Ejaculates were obtained, using a hypodermic syringe, from the uterus of a female rat following copulation.

It has been assumed that the source of the enzyme in the testis homogenate and sperm suspension was similar, i.e., the germ cells.

Hyaluronidase from ejaculates

The previous observation that the enzyme concentration in ejaculates collected from mated female rats was higher than that obtained from testes homogenates (Leonard, Perlman and Kurzrok, 1947) led us to investigate the variation which might occur in enzyme levels *in*

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[2] Fellow of the Schering Corporation.

vitro at varying intervals following copulation. Within two minutes following copulation female rats were killed and the content of the uterus removed as rapidly as possible. The uterine fluid with sperm was diluted to 3–4 cc. with warm Ringer's solution. It was found that best results were obtained when the sperm count exceeded 10 million per cc. A sample was removed, centrifuged, and a hyaluronidase determination carried out on the supernatant fluid. The remainder of the suspension was stored at 37°C. for varying periods and then assayed. In four of five experiments a marked increase with time in

HYALURONIDASE ACTIVITY OF UTERINE CONTENTS FOLLOWING COPULATION

Exp.	Time until removal of cells	Sperm count (millions) per cc.	Turbidity reducing units per cc.	Calc. units per total uterine contents
A	20 min.	—	50	25
	2.5 hrs.		66	33
	13.0 hrs.		90	45
B	20 min.	—	55	27.5
	12 hrs.		80	40
C	10 min.	56	24	6
	1 hr.	56	80	20
D	9 min.	56	<16	<4
	2 hrs.	56	40	10
E	7 min.	18	17	8.5
	1.5 hrs.	18	20	10

the turbidity reducing units (TRU) of enzyme in the supernatant fluid was obsereved (Table 1). Microscopic examination of the sperm showed that a marked decrease in the motility was observed following incubation.

These results suggested that with decreased motility the increased amounts of enzyme were obtained from a dying cell population. Experiments were then devised to increase the enzyme liberated by subjecting the sperm or testes tissue to adverse treatment.

Hyaluronidase level from frozen testis and spermatozoa

If adult rat testes are homogenized soon after removal from the body and extracted in the cold (5°C.) for 30 minutes, the enzyme concentration obtained is 10 TRU per gram (average of more than 30 assays). Rat testes were frozen for 2 and 26 days, then homogenized and extracted. In four experiments, the yield of enzyme was 28, 24, 33, and 30 TRU per gram as compared to the 10 TRU per gram obtained with freshly removed tissue. No great difference was noted when varying the length of time the testes were frozen.

In a similar experiment, sperm suspensions in Ringer-phosphate

solution (pH 6.9) were prepared, using the sperm directly from the ductus deferens and cauda epididymis. One-half the sperm suspension was assayed immediately, the remaining half was alternately frozen and rapidly thawed three times within a period of several hours. The TRU per cc. of supernatant fluid in the control samples was 2, 3, 2, and that from the frozen sperm samples was 10, 10, 6, respectively.

Because previous results showed that incubation of fresh testis homogenate increased the hyaluronidase concentration of the extract (Leonard et al., 1947) *frozen* testes were homogenized and then incubated in order to ascertain whether or not the level of enzyme could be raised further. The incubation of frozen testes increased the TRU per gram of tissue in three experiments to 74, 69, 100 units in comparison with their respective controls (frozen but not incubated) which gave 28, 24, 26 units.

Effect of toluene and acid on testis homogenate

The two testes from each rat were weighed and homogenized separately. To one were added .75 cc. acetate buffer (pH 6.0) and .25 cc. of toluene, the other (control) received 1 cc. of acetate buffer only. After thorough mixing the homogenates were incubated for 3 hours (37°C.). In three experiments the TRU per gram of tissue in the controls were 53, 60, and 67 whereas the toluene-treated testes yielded 115, 110, and 104 units respectively. Furthermore, if the testes were frozen before incubating with toluene, an even higher level of enzyme was secured. The assay of a frozen, incubated testis was 90 TRU per gram; with toluene the other yielded 170 TRU per gram.

In one other experiment, the effect of lowering the pH of the testes homogenate on the subsequent yields of enzyme following incubation was observed. Four drops of 2 N acetic acid were added to one tube containing a single homogenized testis which lowered the pH below 4. The other testis from the same rat was homogenized and 4 drops of acetate buffer added. After 3 hours incubation at 37°C., the extracts were assayed. The TRU per gram obtained from the acid-treated homogenate in three experiments were 46, 56 and 42 units while the controls yielded 40, 51, and 39 units respectively. This indicated no significant increase in liberated enzyme as a result of the treatment with the acid.

DISCUSSION

The first indication that increased amounts of hyaluronidase could be obtained from a dying sperm population was seen in the experiments in which the ejaculates were recovered from the uterus and kept in an incubator at 37°C. for varying periods. At first, this did not appear to be a particularly harsh treatment but the discovery of the decreased activity of sperm suggested that the liberation of the enzyme might well be enhanced under these conditions. Rat sperm do

not remain motile long when removed from the body and suspended in the usual physiological salt solutions. In this respect, rat sperm differ markedly from a number of other mammalian sperm (Chang, 1947).

It is clearly seen that the conditions under which these increased amounts of enzyme were obtained from the testes or sperm were unphysiological and, as such, any interpretation of the assay of hyaluronidase level in tissue or semen samples must be considered in the light of these results. In order to make comparable assays between samples, a rigidly controlled method of handling the samples previous to the assay seems indicated. Presumably, the more resistant the sperm are to adverse conditions the greater will be the likelihood that proportionately lower enzyme levels will be found in the surrounding medium. Whether this is true for all mammalian sperm remains to be determined.[3]

The enzyme-producing cells were given rather drastic treatment in order to determine the quantity of enzyme they were capable of yielding. Acetate buffer is not a good medium for maintaining viability but is the buffer of choice for hyaluronidase assay. Incubation at 37°C. for 3 hours even in what would be considered a favorable artificial environment is not conducive to longevity in rat sperm according to Chang (1947) and Yochem (1930). Freezing and thawing undoubtedly affected the cells in some way, resulting in an increase in the enzyme level of the surrounding medium. Toluene increased the enzyme concentration possibly by its action as a fat solvent in disrupting the integrity of the cell membranes, thereby freeing the enzyme into the surrounding medium (Meyer et al., 1941). In extracting hyaluronidase from skin he suggested also that autolysis of the tissue in the presence of toluene may favor the "conversion of an inactive to an active material." A combination of freezing, incubation and toluene treatment gave, in one experiment, the highest concentration of enzyme ever obtained from rat testes.

A different interpretation may have to be placed on our previous work in which augmentation of the enzyme secured from rat testes could be obtained by adding the seminal vesicle (Leonard et al., 1947). The facts remain unchanged as the experiment has been repeated many times with similar results. Unpublished data also show that augmentation can be demonstrated with suspensions of rat sperm and with the expressed fluid of the seminal vesicle. For the rat at least this also may be unphysiological because most of the seminal vesicle fluid is coagulated to form the vaginal plug and how much of it gains access to the uterus to mix with the sperm is not known.

[3] In a personal communication from Dr. John Mixner, it was pointed out that a similar situation was observed in bull semen samples. After recovery of the ejaculate, increases in enzyme concentration in the seminal plasma occurred with increasing loss of motility of the sperm.

The problem of determining whether living sperm can be induced to increased liberation of hyaluronidase becomes great when the existing criteria for determining living sperm are so inadequate. The degree of motility has been used most frequently but is at best only a subjective method for determining viability. Staining methods for differentiating living from dead sperm have been employed but they are open to criticism as to their specificity.

Since the completion of this work two papers have appeared which bear directly on our results. Hechter and Hadidian (1947) assayed the supernatant fluid obtained from washing rabbit sperm several times and found the level of hyaluronidase similar to that of the original seminal plasma each time. Additional washings, however, revealed a decrease in the amount of enzyme obtained. No mention was made of the change in motility of the sperm during the experiments. Swyer (1947) obtained results similar to ours in observing increased amounts of enzyme by freezing the sperm. He states that sperm do not appear to actively produce hyaluronidase but liberate the preformed enzyme.

SUMMARY

The hyaluronidase level produced by sperm removed from the uterus of a rat and kept at 37°C. was found to increase with time. This was accompanied by a marked loss of motility of the sperm. Using rat testes homogenates and sperm suspensions it was possible to increase the level of this enzyme in the surrounding medium by subjecting the cells to adverse treatment such as freezing, incubation, and toluene but not with acid. These results indicate the importance of establishing the source of hyaluronidase, i.e., living or moribund germ cells, if enzyme assays of semen or tissue samples are to be made.

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VARIABLES AFFECTING THE ASSAY OF INSULIN

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THE INTRODUCTION of the latin square cross-over design and the application of the analysis of variance and covariance to the assay of insulin by Bliss and Marks (1939) was a valuable contribution in providing a means of studying the variables affecting the precision of the method of assay. In order to overcome some of the practical difficulties encountered in the cross-over design Fieller (1940) proposed a split cross-over design which retained many of the features of the former for the segregation of the variables and improved the practicability of the method of assay. Smith et al. (1944) extended the work of Fieller (1940) to the twin cross-over design in order to further simplify the procedure. Lacey (1946) has recently reported observations on the method adopted by U.S.P. XIII and suggested certain improvements with respect to design and specificity. Young (1945) and Young and Romans (1947) have shown that considerable economy of time and effort may be obtained by intravenous administration of the doses of insulin and by limiting the number of bleedings to one instead of the traditional subcutaneous administration employing either four or six bleedings.

This study is concerned with the effect of different bleeding schedules on the efficiency of the assay and with a comparison of the potency of commercial insulin preparations when assayed by the method of Young (1945) and by the method using subcutaneous administration and a bleeding schedule of 0, 1.5, 3 and 5 hours.

METHODS

Rabbits of mixed breed and sex were obtained from the stock colony at 1.5 to 2.0 Kg. body weight in groups of 16 to 20 and were used for periods of six to eight months. The animals were fed a commerical rabbit chow *ad libitum* supplemented with raw carrots twice weekly. Blood samples approximately 1.2 cc. were taken from the marginal ear vein usually after applying a small amount of warm mineral oil to the ear to promote vasodilatation. Sugar determinations were made on 3 cc. of the protein free filtrate according to the method employed by the U.S.P. XIII in the assay of insulin. In the latter part of the work it was found advantageous to lake the blood samples in water, then add the acid zinc sulfate followed by the sodium hy-

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droxide, rather than follow the procedure outlined in the U.S.P. of laking the blood in acid zinc sulfate.

Dilutions for injection were made with U.S.P. XIII diluting fluid adjusted to pH 2.8 to 3.0. The doses for subcutaneous administration were given in a volume of 1.0 cc. per rabbit while in the case of intravenous administration the doses were given in a volume of 2.0 cc. The dosage levels used were adjusted to comply with aperiodic variations in sensitivity and were arranged so that the high dose was twice the low dose, for example 0.90 and 1.80 International Units per rabbit. The effective dosage levels in the intravenous method were within the same range as those required for the subcutaneous method of administration. The doses were given in a random manner according to the 4×4 latin square design. In a few cases where accidents or death of a rabbit interfered with the completion of a square, another rabbit previously treated with insulin was substituted and these results were used in the completion of the square. Preliminary tests were conducted on all new groups of rabbits to accustom them to the bleeding and injection routine and to eliminate animals which appeared highly resistant or sensitive to insulin.

In the assays where subcutaneous administration was employed the rabbits were fasted for approximately 24 hours prior to injection, water was withheld during the test and the animals were used at weekly intervals.

In the case of intravenous administration food was withheld for approximately 16 hours prior to the test, water was available at all times and the rabbits were used daily for four consecutive days. In cases where an initial blood sample was used, this was taken from the marginal vein of one ear and the dose of insulin injected in the marginal vein of the other ear. Each rabbit in the group was injected in this manner at intervals of one or two minutes depending on the ease of bleeding. The final blood sample was taken from the marginal ear vein of each rabbit 50 minutes after the injection. Each day of the test the rabbits were fed after the final bleeding and permitted access to food for approximately 6 hours.

The results of the assays were subjected to analysis of variance as outlined by Bliss and Marks (1939) and the total variance was corrected for differences due to rabbits, days and doses.

RESULTS

The values shown in Table 1 were taken from assays performed using the U.S.P. Reference Standard against commercial samples of insulin prepared from amorphous insulin and zinc insulin crystals. The weighted mean value for the standard error (s) and slope (b) are shown in columns 4 and 5 respectively for each of the bleeding schedules indicated in column 2. In the group using the bleeding times 0 (initial) 1.5, 3 and 5 hours the assays were calculated first on the basis of percentage reduction (the mean decrease of the post injection sugar values as a percentage of the initial value) and secondly as mg. reduction (the mean decrease of the post injection sugar values) in which the initial value was excluded. Similarly the results from the same assays were recalculated to determine the effect of excluding the blood sugar value at 5 hours with and without the initial (0) blood

sugar value. The group of assays using the bleeding schedule of 0, 2 and 4 hours and the group using 0 and 50 minutes were treated in the manner described above. A final group of 24 assays where the initial blood sample was not taken are included for the purpose of comparison.

In order to obtain a measure of one treatment effect in terms of the other, the ratio of $(s/b)^2$ was calculated and is shown in column 6. Due to the relatively small number of assays on each of the treatment effects, the use of different groups of rabbits within and between the

TABLE 1. THE EFFECT OF THE BLEEDING SCHEDULE AND THE METHOD OF ADMINISTRATION ON THE SLOPE OF THE REGRESSION LINE AND THE STANDARD ERROR OF THE ASSAY

No. of assays	Bleeding schedule	Method of adm.	s	b	$(s/b)^2$
10	0,1.5,3 and 5 hrs.	subcut.	7.10	32.69	0.047
10	1.5,3 and 5 hrs.	subcut.	6.09	−28.49	0.046
10	0,1.5 and 3 hrs.	subcut.	7.75	36.91	0.044
10	1.5 and 3 hrs.	subcut.	6.36	−32.62	0.038
9	0,2 and 4 hrs	subcut.	9.21	39.34	0.055
9	2 and 4 hrs.	subcut.	7.49	−35.80	0.044
9	0 and 2 hrs.	subcut.	11.37	42.22	0.072
9	2 hrs.	subcut.	9.41	−37.98	0.061
5	0 and 50 min.	intraven.	11.27	52.53	0.038
5	50 min.	intraven.	9.11	−46.60	0.046
24	50 min.	intraven.	8.50	−40.63	0.044

different bleeding schedules and to the accumulation of the data over a period of three years it was not considered advisable to apply statistical tests for significance to the results, but to consider any difference as indicating trends.

It is seen that the ratio is lower in all cases, except the intravenous method of administration, when the initial value is excluded from the calculations. Similarly the ratio is lower when the bleeding at 5 hours is eliminated. These results indicate that the sugar value at 0 and 5 hours in the bleeding schedule 0, 1.5, 3 and 5 hours may be excluded without affecting the precision of the assay. The assays using the bleeding schedule at 0, 2, and 4 hours were designed to determine if this schedule was more efficient than the schedule using 0, 1.5, 3 and 5 hours. Since the ratio is higher for the former no advantage is indicated, except in reducing the number of bleedings to 3 instead of 4 and shortening the duration of the test one hour. The highest value for the ratio was obtained with the bleeding schedule at 0 and 2 hours. The ratio for $(s/b)^2$ using the intravenous administration and one bleeding at 50 minutes is well within the range of the values found for the subcutaneous administration. Since only one quarter of the blood samples are required in the intravenous method the economy of material and effort is quite apparent and there is considerable saving of time to complete the latin square cross-over design. In this instance

it requires four days as compared to four weeks for the subcutaneous method. The latter method does not permit a feeding period on a test day while in the intravenous method the test can be completed quite conveniently in 2 hours permitting the rabbits to feed for 5 to 6 hours and then withdrawing the food over-night.

The rabbits on each of the treatment effects were weighed at weekly intervals. The results plotted in Figure 1 show that the rabbits

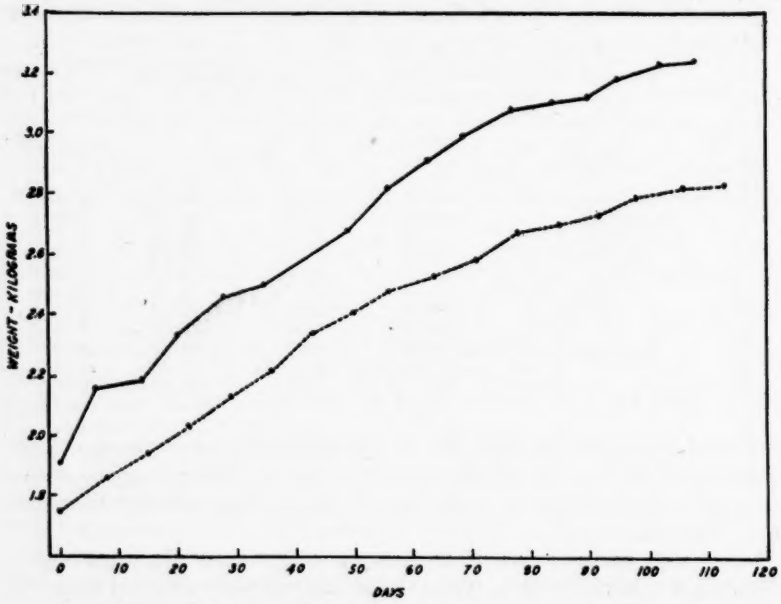

FIG. 1. The effect of the administration of insulin on the body weight of rabbits. Solid line = intravenous injection; broken line = subcutaneous injection. Each point represents the mean body weight of 14 rabbits.

on the intravenous administration gained in weight at approximately the same rate as the rabbits on the sucutaneous method of administration.

The daily bleeding and intravenous injection resulted in no more injury to the ears of the rabbits than was experienced with the subcutaneous method of administration using four bleedings. There was no evidence of the development of tolerance to the intravenous administration of insulin.

Six different samples of insulin supplied by different manufacturers were assayed first by the subcutaneous method using the bleeding schedule at 0, 1.5, 3 and 5 hours and secondly, by the intravenous method using one bleeding at 50 minutes. The results in Table 2 show

TABLE 2. A COMPARISON OF POTENCY OF COMMERCIAL SAMPLES OF INSULIN ASSAYED BY SUBCUTANEOUS ADMINISTRATION AND BLEEDINGS AT 0,1.5,3 AND 5 HOURS WITH INTRAVENOUS ADMINISTRATION AND BLEEDING AT 50 MINUTES

Sample No.	Labelled potency in I.U.	Subcutaneous				Intravenous			
		b	s	$(s/b)^2$	Potency found in I.U.*	$-b$	s	$(s/b)^2$	Potency found in I.U.*
1	20	44.12	7.69	.030	*21.8* (17.9 to 26.6)	40.53	6.46	.025	*20.4* (17.0 to 24.4)
2	20	41.76	6.29	.023	*20.6* (17.1 to 24.1)	49.14	6.81	.019	*21.1* (18.0 to 24.7)
3	20	33.29	7.30	.048	*23.2* (18.0 to 29.9)	33.22	8.05	.059	*20.4* (15.6 to 26.6)
4	20	24.65	7.04	.081	*21.1* (15.3 to 29.1)	40.26	7.57	.035	*21.1* (17.0 to 26.1)
5	40	32.22	5.54	.029	*36.6* (29.9 to 44.8)	46.37	7.57	.026	*31.5* (25.9 to 38.2)
6	40	22.03	6.49	.086	*38.4* (27.5 to 53.5)	35.35	7.10	.040	*42.4* (33.7 to 53.2)

* Mean potency in italic type, range expressed as $1.96 \times Sm$.

there is good agreement in the estimation of potency and in the precision of assay by the two methods. The value (b) for the slope of the regression line in the case of the intravenous method, where mg. reduction is used as the response, is negative on account of the inverse relationship between dose and response.

DISCUSSION

The majority of the reports on the assay of insulin have been concerned either with the effect of variations in the design or with methods of interpreting the assay results without much consideration of the bleeding schedule. Recently DeJongh et al. (1947) have reported their results on the correction factor for the initial blood sugar. They have shown that equally satisfactory results can be obtained by correcting the initial sugar determination and basing the results on the absolute value of the decreased blood sugar, or by expressing the results in terms of per cent of the initial value. In both cases they found a highly significant correlation between the initial blood sugar and the reduction to be obtained after subcutaneous administration and a bleeding schedule at .75, 1.5, 2.25 and 3 hours after injection. The results presented in Table 1 indicate that the uncorrected values for the initial blood sugar do not contribute to the precision of the assay, and in the interests of efficiency may be omitted. Admittedly it would be advisable to conduct control blood sugars on new groups of rabbits in order to rule out any that show abnormalities in this respect. Since the labor and expense involved in the assay of insulin are relatively greater than in most assay procedures, any reduction in these factors appear to be justified. In this connection Bliss and Bartels (1946) in a preliminary report using the discriminate function test claim that the different time intervals in the bleeding schedule of the insulin assay are

markedly unequal in the information they provide on the potency of the product. Although the results indicate that the 5 hour sugar value does not contribute to the precision of the assay, however it is of value in providing qualitative information on the reaction of the rabbit to insulin. In common with most biological assays this method leaves much to be desired with respect to the use of the product clinically, for the rates of absorption and utilization of insulin in the diabetic individual is undoubtedly different from these rates in the normal rabbit. Hence, the justification for a method of assay that ensures uniformity of a product. Thus far no attempts have been made to correlate the rabbit assay method with a method using diabetic patients.

The method of assay reported by Young (1945) and Young and Romans (1947) using intravenous administration of the doses and one bleeding 50 minutes after injection gives results (Table 2) in good agreement with those obtained by the subcutaneous method of administration. The economy of time and effort of the former is shown in Table 1. The method was found very practical and no untoward effects were observed in the rabbits. Normal weight gain was maintained (Figure 1) and it has been our experience that the incidence of convulsions in assays is considerably lower than with subcutaneous method of administration. Young (1945) has studied the dosage response relationships and has shown that the method as outlined above complies with accepted principles of a satisfactory assay. The two dosage levels of standard and sample employed in this work yield a limited amount of data on this point, but in all the assays reported here a significant slope was found and in no case was there evidence of non-parallelism between standard and sample. Young (1945) made a preliminary study of bleeding times at 45, 60 and 110 minutes after injection and the results indicate that these bleeding times do not offer any advantage over the bleeding schedule at 50 minutes. The latter is a very practical time in the conduct of the assay, but undoubtedly this point deserves further study.

SUMMARY

In the assay of insulin using rabbits the results indicate that the efficiency of the method can be considerably improved without loss of precision by omitting the initial and final blood sugar determinations in the conventional bleeding schedule. Considerable economy of time and effort without loss of precision can be accomplished in the assay of insulin by intravenous administration of the doses and using one bleeding 50 minutes after injection. The potency of commercial insulin preparations when assayed by the intravenous administration of the doses showed excellent agreement with the potency determined by the subcutaneous administration of the doses and a bleeding schedule at 0, 1.5, 3 and 5 hours.

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EFFECT OF RECIPROCAL STEROID TREATMENT ON THE ELECTROPHORETIC PATTERNS OF FOWL SERA

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MARKED differences in the electrophoretic patterns of cock and hen sera were reported in 1945 (Moore). It is the purpose of this communication to trace the development of these differences beginning with the young chick and to report the changes produced by injecting the adult fowls with contra-sex hormones.

METHODS

Blood was taken from the heart by inserting a syringe needle in front of the furculum into the thorax. By this method 5 to 10 cc. of blood could be taken without apparent injury to the bird. The sera were analyzed in a buffer 0.02 M with respect to sodium phosphate and 0.15 M with respect to NaCl at pH 7.4 (Tiselius and Kabat, 1939). Each serum sample was diluted with two parts of the buffer and dialyzed in a bag made from viscose sausage casing (average pore diameter 2.5 mμ) against one liter of the buffer for 24 hours, whereupon the volume was re-measured in order to correct for any change during dialysis. The conductivity and pH of both the diluted sera and dialysate were routinely taken. The conductivities were used in calculating the mobilities of the various electrophoretic components.

The analyses were carried out in a Tiselius apparatus (Tiselius, 1937) having a single-sectioned tall cell of 2 cc. capacity. The center section is 50 mm. tall, 15 mm. deep (along the optic path) and each channel is 2 mm. wide. The channels are 10 mm. apart (center to center). A current of 18.7 milliamperes flowed through the cell and produced a field strength of 6.5 volts/cm. All patterns were obtained by the scanning method of Longsworth (1939) on panchromatic plates (Kodak M), the light source being a 200 watt tungsten projection lamp. If photographed with uniform exposure, the area of a pattern component is proportional to the product of the specific refraction and the concentration of the corresponding serum component.

EXPERIMENTAL

The chickens used in these experiments were Rhode Island Reds. Distinct sex differences in the serum patterns were not apparent until the fourth or fifth month of life, hence the hormone experiments were not begun until the 175th day of life, which was after the hens had started laying. At this time 2 cocks were injected intramuscularly

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with 0.1 mg. α-estradiol dipropionate (Di-ovocylin, Ciba) in sesame oil and 3 hens were injected with 0.2 mg. testosterone propionate (Perandren, Schering) daily for 14 days. The sera immediately before and after this treatment were compared.

After several weeks the experiments were continued with larger doses of hormones and young adult capons were substituted for the cocks. Two of the hens received 1 mg. testosterone propionate daily for 18 days, while the third received an equivalent volume of sesame oil as a control. The three capons, after being bled for control sera, received 1 mg. Di-ovocylin per day for 18 days.

Some samples of sera were extracted with ether by the method of McFarlane (1942). Two volumes of serum were mixed thoroughly with 1 volume of chilled ether and quickly frozen in a dry ice-alcohol bath. The sample was then allowed to thaw and separate in the ice box for 8 hours or longer, whereupon the serum was carefully removed from underneath the ether layer with a syringe and blunt needle. This process was repeated two additional times. Residual ether was removed by a short period of vacuum desiccation. McFarlane reports that this process removed only part of the total lipids but apparently does not denature the proteins. A comparison of nitrogen (determined by micro Kjeldahl method) with pattern areas before and after ether extraction indicated that the ratio of pattern areas to nitrogen was reduced by 15 to 25 per cent by the extraction process. This result shows that a large quantity of material having a high refractive index to nitrogen ratio was removed by the ether.

OBSERVATIONS

The data of Tables 1 and 2 are summarized in the patterns of Figure 1. It is apparent that pronounced sex differences in the sera do not appear in the chicken until maturity and become more pronounced with age. The hens in these experiments began laying between the 155th and 170th day. The administration of 0.1 mg. androgen daily for 14 days in the hen did not produce significant changes in the serum except that the component of highest mobility was reduced. This component was never present in cock serum and disappeared upon extraction with ether in hens. Administration of 0.2 mg. estrogen for the same period in the 175 day old cocks did, however, produce appreciable changes in the serum. Component 2 virtually disappeared, the fastest moving component began to appear and the lagging end of the pattern took of the spikey character of a hen serum pattern (see Figure 1, low hormone injected). Encouraged by these results the daily injections were later increased to 1 mg. and adult capons were substituted for the cocks. At the beginning of this latter experiment the hens were 260 days old and had resumed laying some days after cessation of the 0.1 mg. androgen injection. During this rest period the lagging components of the serum pattern had greatly increased in size assuming the

TABLE 1. ELECTROPHORETIC DATA ON NORMAL HEN AND COCK SERA AT VARIOUS AGES

Identification	Pattern Areas (Arbitrary units)							Mobilities*					
	f	1	2	3	4	5	Total	f	1	2	3	4	5
Pullets and Cockerels at 42 Days and 84 Days of Age													
42da ♀		195	50	40	35	25	345		5.2	4.4	3.4	2.1	1.3
42da ♂		180	70	35	50	40	375		5.1	4.1	3.2	2.4	1.2
84da ♀		140	95	25	95		355		5.2	4.3	3.4	2.5	
84da ♂		185	80	25	80		370		5.3	4.3	3.4	2.6	
100 Day Pullets													
#2		235	70	20		190	515		5.0	4.2	3.1		1.6
#4		250	80	35	105	45	515		5.0	4.2	3.1	1.8	1.1
#6		220	70	15	80	30	415		5.0	4.2	2.9	1.8	1.2
100 Day Cockerels													
#1		215	95	30	150		490		5.0	4.2	3.1	1.8	
#3		180	100	20	120		420		5.0	4.2	3.1	1.8	
#5	5	205	100	40	70	30	450	6.0	5.0	4.2	2.9	1.6	1.0
135 Day Pullets													
#2		210	60	30	125	75	500		4.7	3.8	2.9	1.6	1.0
#4		180	70	25	50	40	365		4.8	3.9	3.0	2.4	1.2
#6		215	95	45	140		495		5.0	4.2	3.3	2.3	
135 Day Cockerels													
#1		230	120	50	100	35	535		5.2	4.2	3.3	1.8	1.1
#3		160	100	30	65	25	380		5.2	4.2	3.3	2.0	1.5
#5		235	110	45	80	35	505		4.9	3.9	3.0	2.4	1.2
175 Day Hens													
#2	90	220	30	40	270		750	6.3	5.1	4.2	3.1	1.8	
#4	45	335	25	65	100	85	655	6.5	5.2	4.2	3.1	1.9	0.5
#6	45	340	15	25	170		595	6.1	5.1	4.2	3.1	1.8	
175 Day Cocks													
#3		180	95	20	90	20	405		5.2	4.2	3.2	2.2	1.8
#5		170	75	30	120		395		5.3	4.4	3.4	1.9	
175 Day Hens—Ether Extracted													
#2		270		30	105	75	480		4.8		3.0	1.7	1.2
#6		290		30	115	20	455		4.8		3.1	1.9	1.0
175 Day Cocks—Ether Extracted													
#3		205		60	40	10	315		4.9		3.1	1.8	1.1
#5		125		70	40	20	255		4.9		3.0	1.8	1.1

* Mobilities derived from descending patterns are expressed in units of 10^{-5} cm.²/volts-sec. All components are anodic.

proportions previously reported (Moore, 1945) for mature laying hens (see Figure 1, control). After 18 days of the high androgen administration, the hen serum pattern was almost identical to the typical pattern of cock serum. The fastest component had receded, a normal cock serum component 2 had appeared and the lagging portion of the pattern had diminished and taken on the contour of a cock serum pattern. Similarly, the capon serum which was already different from normal cock serum, was almost transformed into a typical hen serum

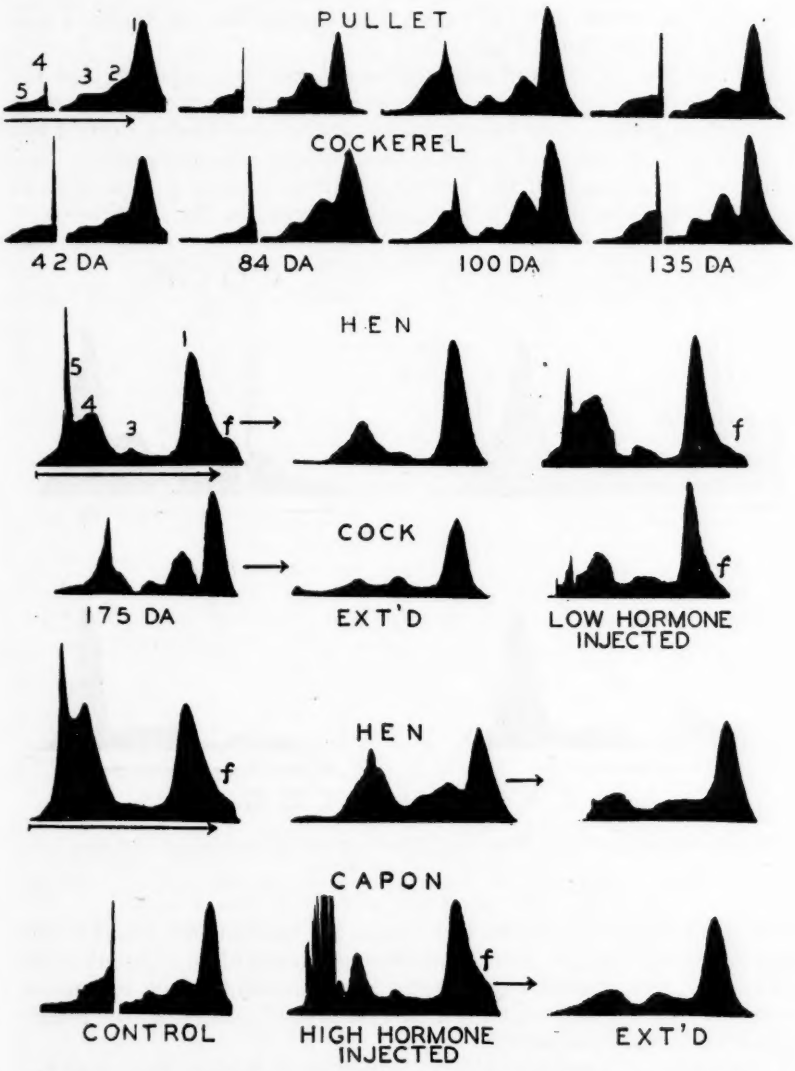

FIG. 1. Electrophoretic patterns of female and male chicken sera showing similarities during the development period (up to 135 days); difference after maturity (175 days), same sera ether extracted (ext'd), changes produced by 0.2 mg. testosterone propionate and 0.1 mg. α-eatradiol dipropionate daily for 14 days in the hen and cock respectively (low hormone); control hen and capon at 260 day (control); after 1 mg. per day in both hen and capon for 18 days (high hormone), and same sera ether extracted (ext'd).

(cf. high hormone injected capon with control hen of Figure 1 and also with laying hen of Figure 2).

From the patterns of ether-extracted sera it is apparent that the substance responsible for sex difference is largely removed by the ether extraction process. The fastest component in the 175 day normal hen serum and component 2 in the normal cock serum disappeared, leaving the leading ends of the pattern identical in respect to number of components. Component 3 was larger, however, in the extracted cock

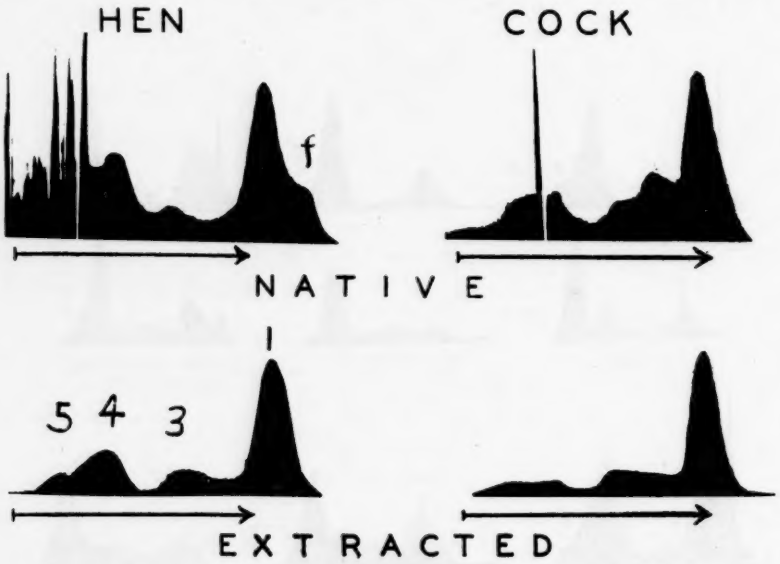

FIG. 2. Laying hen and normal cock serum patterns before (native) and after (extracted) ether extraction.

serum than in the extracted hen serum, but components 4 and 5 were much larger in the hen serum, component 5 being almost absent in the extracted cock serum. After hormone treatment ether extraction rendered the hen and capon sera almost indistinguishable (see Figure 1, high hormone, extracted).

In order to ascertain that the reduction in pattern area was not accompanied by a similar reduction in protein nitrogen, a comparison of electrophoretic pattern area with nitrogen content was made before and after ether extraction. The data are presented in Table 3. Kjeldahl measurements were made on the diluted and dialyzed material that went into the electrophoresis cell. The ratio of pattern area to nitrogen content was reduced more in laying hen serum than in normal cock serum.

TABLE 2. ELECTROPHORETIC DATA ON HEN AND COCK SERUM AFTER STEROID TREATMENT

Identification	Pattern areas, Arbitrary units							Mobilities*					
	f	1	2	3	4	5	Total	f	1	2	3	4	5
190 Day Hens After 0.2 mg. Testosterone Propionate Daily for 14 Days													
#2	50	285	25	50	180	130	720	5.6	4.6	3.4	2.9	1.6	1.1
#4	20	250	30	30	55	95	480	5.5	4.8	3.6	3.0	1.8	1.5
#6		260	55	55	170	35	585		5.1	4.0	3.3	1.8	1.4
190 Day Cocks After 0.1 mg. α-estradiol Dipropionate Daily for 14 Days													
#3		155	50	30	100	20	355		5.0	4.1	3.2	1.9	1.4
#5		160	90	30	65	20	365		5.0	3.9	3.3	1.9	1.4
Hens After 1.0 mg. Testosterone Propionate Daily for 18 Days													
#2C†	60	280		50	270	245	905	5.9	5.0		3.2	1.9	1.1
#4		200	75	55	210		540		5.0	3.9	3.2	1.9	
#6		205	50	40	180		475		4.9	4.0	3.2	1.9	
Capons—Before and After 1.0 mg. α-estradiol-Dipropionate Daily for 18 Days													
#A Before		220	70	20	45	80	435		5.2	4.1	3.3	2.0	1.3
#A After	10	250	40	35	100	90	525	6.0	5.0	3.9	3.0	1.8	1.2
#B Before		210	70	25	50	50	405		5.2	4.2	3.2	2.1	1.3
#B After	20	220	60	50	125	150	625	6.1	5.4	3.9	3.4	2.1	1.4
#C Before		190	65	30	80	50	415		5.1	4.2	3.2	2.6	1.4
#C After	30	280		75	130	240	755	6.1	5.1		3.2	2.1	1.5
Ether Extracted After 1 mg. Hormone Daily for 18 Days													
♀ ~~#6-4~~		215		60	65	20	360		5.2		3.4	1.7	1.1
Capon #A		220		60	100		380		5.0		3.2	1.8	

* Mobilities derived from descending patterns are expressed in units of 10^{-5} cm.²/volts-sec. All components are anodic.

† Control injected with sesame oil without hormone.

TABLE 3. COMPARISON OF SERUM ELECTROPHORETIC PATTERN AREAS WITH KJELDAHL NITROGEN BEFORE AND AFTER ETHER EXTRACTION

Serum	Pattern area-arbitrary units							Total nitrogen Kjeldahl mg/cc	Pattern area to nitrogen ratio	Change in ratio %
	f	1	2	3	4	5	Total			
Normal Laying Hen										
Native	75	270		50	130	220	745	2.45	305	
Extracted		240		45	60	35	375	1.59	235	−23
Normal Adult Cock										
Native		250	85	40	70	50	495	1.87	265	
Extracted		255		65	25	5	350	1.58	220	−17

DISCUSSION

Sex differences in the electrophoretic pattern of fowl sera have been confirmed. Deutsch and Goodloe (1945) state that "a slight difference between male and female plasmas was apparent." The differences previously reported from this laboratory (Moore, 1945) and those reported now show marked sex differences. The failure of Deutsch and Goodloe to observe significant sex differences may have been due to their employment of a barbiturate-sodium citrate buffer at pH 8.6 or they may not have obtained plasma from fully mature birds.

The comparison made here of chicken serum before and after ether extraction illustrates the enormous quantity of lipoid substance it contains, and also indicates that the sex differences observed in the electrophoretic patterns are largely removed upon ether extraction. Zeldis, Alling, McCoord and Kulka (1945) have shown that the removal of lipids by an alcohol-ether extraction method described by Blix (1941) reduces the electrophoretic pattern area to protein nitrogen ratio in both human and dog plasma. In hyperlypenic dogs the reduction was principally in the α-globulin, although the pattern area of all the components was usually reduced. β-globulin, however, was the principal loser in the several pathological human plasmas examined. McFarlane (1942), in his studies on normal human serum, reported that by the method of lipid extraction used in this work only β-globulin is reduced. In chicken serum, however, this ether extraction process reduces the area represented by several electrophoretic components and causes some of them to disappear completely (e.g., the f-component in hen and the α-component in cock sera). Not only lipids but also some protein is removed as is evidenced by the decrease in nitrogen after extraction (see Table 3). This probably indicates that the sera contain firmly bound lipoprotein complexes which are predominantly lipid and therefore extractable by the method used.

The rapid increase in pattern area (see Table 1, 135–175 days) as the hen begins to lay is consistent with the increase in body fat at this period, reported by Halnan and Cruickshank (1933). In view of the researches of Zondek and Max (1939), Lorenz (1938 and 1943) and Flock and Ballman (1942), which demonstrated artificially-induced rapid fattening of male chickens by administration of estrogens, it is not surprising that serum from normal hens or estrogen-injected cocks or capons contained a large amount of lipid. However, the pattern for normal adult cock serum is also appreciably reduced upon ether extraction, and component 2 completely disappears, indicating that lipids or lipoproteins may be present in components not presenting a sharp or spiked pattern.

SUMMARY

Sex differences in the electrophoretic pattern of chicken serum appeared at the beginning of sexual maturity. Hen and cock serum

patterns were reversed by contra-sex hormones. Ether extraction indicated a high lipoprotein content of both cock and hen sera which was mainly responsible for the electrophoretically-observed differences.

ACKNOWLEDGMENTS

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ANATOMICAL COMPARISON BETWEEN THE ADRENAL GLANDS OF WILD NORWAY, WILD ALEXANDRINE AND DOMESTIC NORWAY RATS[1]

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OUR KNOWLEDGE of the effects produced by domestication on the endocrine system of animals is very limited, owing in large part to the fact that domesticated and wild forms of the same animal are rarely available simultaneously. The common Norway rat, however, constitutes a definite exception to this statement, since it has many representatives of both domestic and wild forms alive at the present time. Domestic Norways have been reared in laboratories throughout the world for the past 60 years or more, while the wild Norways still live in alleys, yards, cellars and houses and also have a world wide distribution. The domestic rats are tame, tractable, do not attempt to escape, and reproduce well in captivity; the wild rats usually live in burrows in the ground and are fierce, savage, suspicious, and make every effort to resist captivity. Except under particularly favorable conditions, they have not been successfully reared in captivity.

The common Norway rat thus provides excellent material for studying the effects that are produced by domestication. From the great variety of experiments carried out over such an extended period of time we probably know more about the domestic Norway rat than about any other animal. However we know very little about its wild relatives even though they are available for study in large numbers almost at our doorstep.

Until recently the lack of a simple method of catching wild Norway rats alive and in large numbers prevented the carrying out of such a study. During the war, when large numbers of wild rats were needed for screening of rodenticides, we put into use an inexpensive, easily operated trap that made it possible to catch wild Norway rats with considerable ease and certainty (Richter and Emlen, 1945).

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[1] This work was begun under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Johns Hopkins University, and completed under a grant from the Rockefeller International Health Board for work on the biology of the wild rat.

[2] On leave of absence 1945–1946 from Hamilton College, Clinton, New York.

Several comparative studies on the anatomy, pharmacology and physiology of these two strains have already revealed marked differences between the domestic and wild forms (Dieke and Richter, 1945; Fish and Richter, 1946; Griffiths, 1944 and 1947). The present study concerns the effects produced on the adrenal glands.

H. H. Donaldson in 1924 reported that recently trapped wild rats had heavier adrenals than did his domestic rats. In 1928 J. C. Donaldson reported that an increased amount of cortical tissue accounted for this difference.

We have made a more extensive study of the adrenals in these two strains: (1) on growth of adrenals with relation to body weight; (2) on relative weights of cortex and medulla of wild and domestic forms and the effects produced on these relative weights in wild rats by extended periods in the laboratory on a regular laboratory diet; (3) on changes that result from crosses between wild and domestic rats.

For purposes of further orientation in adrenal size of wild rats, studies were made also on the wild Alexandrine rat. This animal, next to the wild Norway, is the most common rat throughout the world. Compared to the Norway it has a longer tail, larger ears, and a sleeker and more streamlined appearance. It inhabits ships, seaport towns, and cities, and tends to live and make its nests in ceilings and rafters rather than in burrows. This rat has not been domesticated. It was not possible therefore to obtain any data relating to the domestic form of this animal.

MATERIAL AND METHODS

Our colony of domestic Norway rats originally came from the Wistar Institute. Since then, except for a few pigmented animals added in 1927, no rats from other strains have been introduced. The wild Norways and most of the Alexandrines, other than those designated as being born in the laboratory, were trapped in the city of Baltimore and examined as soon as they were received. A few of the Alexandrines came from Florida.[3]

To detect any seasonal effects, we made a separate comparison of the adrenal weights of wild Norways killed in the summer and in the winter months.

The adrenal glands were dissected out immediately after death and weighed on a torsion balance to the nearest tenth of a milligram.

A total of 107 domestic Norways, 147 wild Norways and 69 wild Alexandrines were used to obtain the adrenal growth curves. In each instance these rats were evenly distributed over the entire body weight range (none below 25–50 grams).

The relative weights of cortex and medulla could not be determined directly because an accurate separation of the adrenal into its constituent parts for weighing is impossible. An indirect method was therefore resorted to, namely the comparison of the wieght of paper cut to conform with outlines of the cortex and medulla as projected from histological sections of the glands.

[3] J. Spencer of the Fish and Wildlife Service at Gainesville, Florida, kindly trapped and sent these rats to us.

After sectioning, at 20μ, and staining with hematoxylin and eosin, every fifth section was projected by means of an Edinger projection machine onto No. 3 Green Spring bond paper and the outlines of both the cortex and medulla were traced at a magnification of 45× (Jackson, 1919, modified). A total of more than 60 projections was thus obtained for the smallest set of adrenals and many more for those of the larger rats. The tracings were cut out and all weighed on the same day to avoid errors which might arise from variations in atmospheric conditions. The medullary parts of each tracing were then cut free from the cortex and weighed again. The relative percentages of the medulla and cortex were thus determined. The actual weight of each part was then calculated from the known weight of the whole gland.[4]

For the determination of the cortico-medulla relationship we used 2 males and 2 females from each of the following 5 groups: domestic Norways, wild Norways killed soon after capture, wild Norways born in the laboratory and kept under laboratory conditions for about a year, wild Alexandrines killed immediately after being received from Florida (2 males and 1 female), and wild Alexandrines born in the laboratory and kept for about a year. The exact age of the wild freshly trapped rats could not be determined, but by comparing the weights of the animals caught in the field with those of known age brought up in the laboratory, we have estimated that the wild Norways were one year old or a little less. We took into consideration the fact that wild rats living under laboratory conditions are generally heavier than those living in the wild state and that a domestic rat tends to be fatter than a wild one brought up in the laboratory. The domestic Norway males used weighed in the neighborhood of 300 grams; the wild Norways from the laboratory 250 grams; and those from the street 225 grams. The Alexandrines born in the laboratory averaged 225 grams and those from the street 150 grams. The females weighed slightly less than the corresponding males; none of them was either pregnant or lactating. Only rats in apparent good health were utilized, as it is well known that infection will greatly increase the size of the adrenal cortex (Grollman, 1936).

RESULTS

The photographs of adrenal glands in Figure 1 show the effect that domestication has on the size of this gland. In typical female animals weighing about 250 grams the left adrenal of the domestic Norway weighed 32.4 mg. as compared with 67.0 mg. in the wild Norway and 65.6 mg. in the Alexandrine. The great discrepancy in size is even more striking in 350 gram animals; at this weight the left adrenal illustrated for the domestic Norway weighed 37.4 mg., while that of its wild counterpart weighed 108.0 mg. A similar difference in size is shown in the males.

Growth curves. Figure 2 shows the combined weight of both adrenals in relation to body weight in male and female rats of the domestic and wild Norways, and wild Alexandrines. Adrenal weights were obtained

[4] Since this method gives relative volumes rather than weights, it had to be assumed, in reducing to a weight basis, that cortex and medulla had the same density.

for 65 male and 42 female domestic rats (weighing from 30 to 430 grams); 68 male and 79 female wild Norways (weighing from 50 to 580 grams); and 38 male and 31 female Alexandrines (weighing from 25 to 220 grams). The straight lines shown were then fitted to these data by the method of least squares.

The adrenals of the wild Norway and Alexandrine rats of both sexes weighed far more than did those of the domestic Norway, particularly in the higher body weight ranges. At 100 grams body weight the adrenals of the male wild Norway weighed twice as much as did

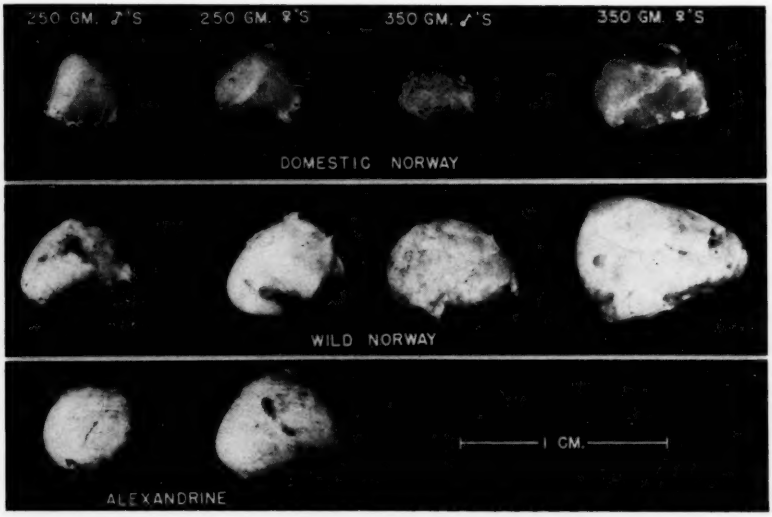


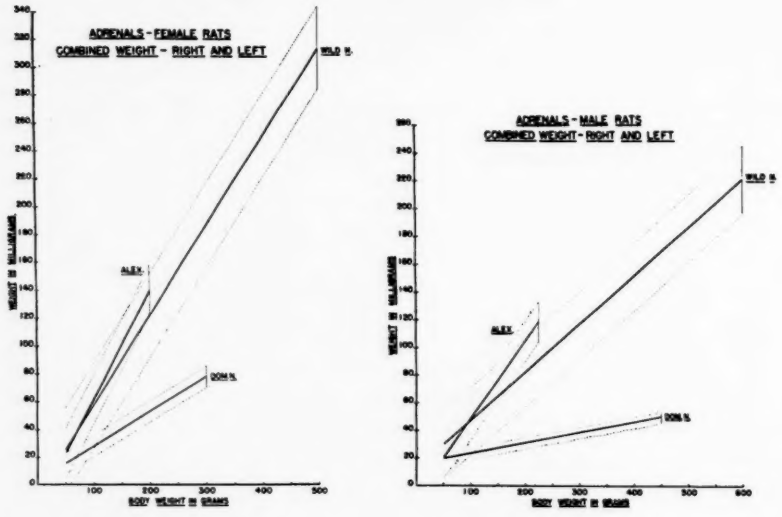
FIG. 1. Photographs of typical left adrenals of male and female domestic Norway, wild Norway and Alexandrine rats at two body weights.

those of the male domestic Norway and more than three times as much at 400 grams. The adrenals of the male Alexandrine were even heavier than those of the wild Norway at a corresponding body weight. There was no significant difference between the adrenals of the female wild Norway and Alexandrine.

Sex differences. Adrenal weights of male and female domestic Norway rats under 100 grams showed no marked differences (Fig. 2). Above 100 grams, however, the weights of the female adrenals were larger than those of the corresponding males. Thus, at 150 grams, the males had adrenals averaging about 25 mgs. and the females 40 mgs. At 300 grams body weight the males had glands averaging 38 mgs., while the glands of the female were twice that size.

This sex difference was also pronounced in the wild Norway rat. Figure 2 shows that, as in the domestic animals, adrenals were far

heavier in wild females than in wild males of the same body weight. The divergence, however, starts at a lower body weight than in the domestic animals. Thus, at 100 grams the glands weighed 65 mgs. in females and 48 mgs. in males, while in the domestic rats the glands weighed 27 and 22 mgs. respectively. At 400 grams the glands of the wild rats weighed 250 and 143 mgs. respectively.



A B

FIG. 2. Variation in weight of both adrenals with body weight for domestic Norway, wild Norway and wild Alexandrine rats. Straight lines fitted to data by method of least squares. Dotted lines (at plus and minus one standard error) indicate degree of scattering around the lines: 68% of the individual values fall within the dotted lines in each case. A. Female rats. B. Male rats.

In contrast, Alexandrine rats show little or no sex difference in adrenal weights. Figure 2 shows that even in 200 gram rats, which are large for their species, the females had adrenals weighing only slightly more than those of the males.

Seasonal changes. The adrenals of wild Norway rats caught in the warm summer months were smaller than those taken during early winter. The seasonal difference is far more marked in the male than in the female. Thus, in 350–400 gram rats the adrenals of males killed in the winter months averaged about 50 per cent heavier than those taken during the summer. In the females, the difference was too slight to be statistically significant. Figure 2 gives data for rats caught in the winter.

Domestic wild Norway crosses. Eleven male and 11 female offspring from wild Norway rats bred to black domestic animals were employed

for this study. At the age of 6 months, when sacrificed, the 11 males weighed from 325–392 grams and the 11 females 215–275 grams. The average weight of the adrenals in the males was 61.2 mgs. or only slightly more than that of domestic males in the same weight group (40 mgs.). Corresponding wild Norway males, however, had adrenals weighing about 138 mgs. (see Fig. 2). The average weight was 75.0

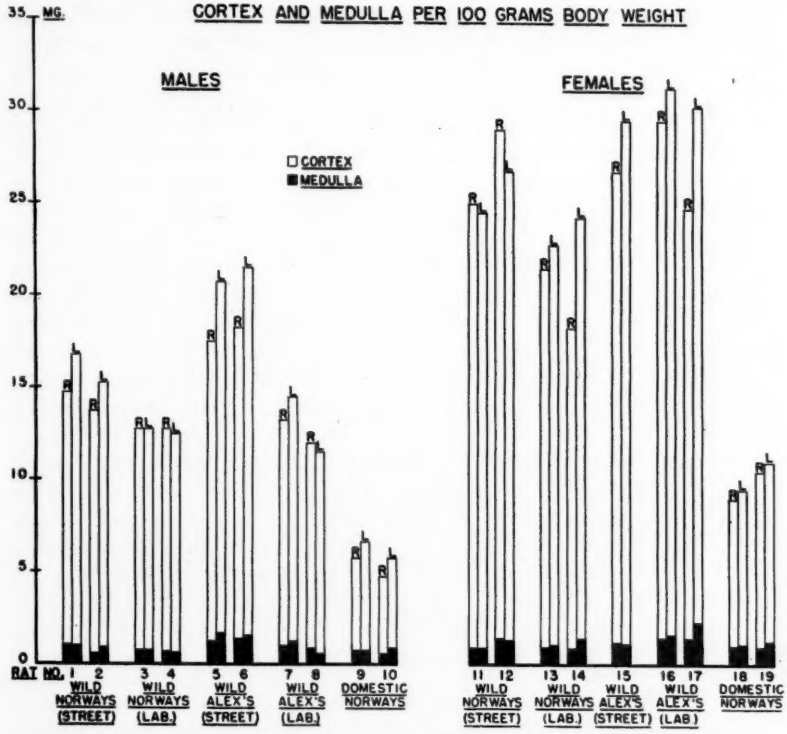


FIG. 3. Relative amounts of cortex and medulla found in the individual adrenals (left and right) of domestic Norway rats and of wild Norway and Alexandrine rats living under street or laboratory conditions. For purposes of comparison all data are given in milligrams per 100 grams of body weight.

mgs. for the females, compared with 64.0 mgs. for the domestic and 220.0 mgs. for the wild Norway females of corresponding body weight. Thus, the adrenal weights of rats of the first generation-crosses approximate those from the domestic rather than the wild parent.

Cortex-medulla relationship. Figure 3 gives the weights of the cortex and medulla, in mg. per 100 gram body weight, for both glands. This was determined in 19 rats by the combined histological and paper drawing methods.

The chart shows that a great reduction in the weight of the cortex accounts almost entirely for the smaller adrenal weights in the domestic rats as compared to either of the wild forms. In general the wild and domestic Norways had essentially the same medulla weights; the wild Alexandrines had heavier medullas. The females had larger medullas as well as cortices. The 12 month stay in the laboratory in general reduced the cortex weights, but the results are not consistent.

Histological differences. The adrenals of the wild rats had thicker fasciculata and reticularis. In all three kinds of rats the fasciculata was a slightly thicker band than the reticularis. The only marked cytological differences observed in the glands (regularly fixed in Bouin's solution and stained with hematoxylin and eosin) was in the juvenile cortex. However, no special fixatives or stains were used in this study. The fasciculata-like cells of the juvenile cortex, when present, were between the inner layer of the cortex and the medulla or embedded as extensions into the medullary material. These cells had about the size of the typical fasciculata cell but showed no evidence of vacuoles. The more usual arrangement was a tightly packed group forming either large or small islands in the inner region of the reticularis or between it and the medulla. This was true in both the street and laboratory wild Norways and to a lesser degree in laboratory Alexandrines. In the animals studied the islands of the latter group were infrequent and small. In contrast, the street Alexandrines, both male and female, showed a band of connective tissue, either wide or narrow, entirely surrounding the medulla. The juvenile cortical cells were embedded in this connective tissue band. In the case of the Alexandrine female the broad band of connective tissue and juvenile cortical cells was extremely vascular, far more so than the main part of the reticularis layer. These cells were present in incomplete bands between the cortex and medulla in the domestic Norway. However, the connective tissue, so striking in the Alexandrine, was entirely lacking.

DISCUSSION

The findings reported above demonstrate that one of the marked changes undergone by the Norway rat during its many generations of laboratory life, is a great decrease in the size of the adrenal glands in both sexes. Watson (1907) pointed out many years ago that wild rats after ten weeks of captivity had smaller adrenals than those killed in the field. Our study bears out this finding in that the adrenals of the Norways living under laboratory conditions were slightly smaller than those of rats fighting for their existence outside the laboratory, however, still not as small as adrenals from domestic animals. This decrease in the relative amount of adrenal tissue may be only an apparent one, owing to the fact that a wild rat living under laboratory conditions frequently accumulates fat. A larger quantity of fat would increase the body weight and so make the same size adrenals appear

to weigh less, when expressed per 100 grams of body weight. It is not known, however, whether or not this increase in fat is offset by an increase in muscular tissue in the street rat.

The great discrepancy in adrenal size is confined entirely to the cortex, as there is little or no change in the absolute amount of medullary tissue. Rats living a protected life in the laboratory where they are unaccustomed to great stress and shock apparently have no need for large amounts of cortical tissue. Griffiths (1944) has pointed out that domestic rats subjected to high frequency sounds have fits while the wild rats, both Norways and Alexandrines, are unaffected. We have found that while domestic rats survive adrenalectomy and, when given salt solution, continue to gain weight, wild Norways die within 12 days even when receiving salt in their drinking water or food (unpublished data).

The relation between the adrenal cortex and shock has been pointed out by numerous investigators who have shown that many forms of shock will cause adrenal hypertrophy. Short exposure to cold (Hartman, et al., 1931), stress (Ingle, 1943), vitamin E deficiency (Blumenfeld, 1934), morphine (MacKay, 1931), and numerous other drugs (Selye, 1937), cause an increase in the adrenal size of domestic rats. Adrenal hypertrophy is one of the changes taking place during the "alarm reaction" (Selye, 1936). However after shock the domestic rat adrenals never reach the size attained in the wild Norways. Selye (1937) reports a 50 per cent increase in adrenal weights of 90-day old rats after atropine, and MacKay (1931) has shown that acquired morphine tolerance will enlarge the adrenals as much as 70 per cent.

There is no evidence to show whether adrenal cortical hypertrophy is associated with hyperactivity. Flexner and Grollman (1939) have demonstrated that an increase in the cortical lipoid content of domestic rats tends to be correlated with greater activity but the complete "significance of the lipoid content of the cortex in relationship to functional activity is not definitely established" (Ingle, 1942). Further work must be done on the secretions of the cortical cells of the domestic rat and a comparison made between the quantitative output of these secretions and the activity of the two varieties of rats before it can be determined whether the decrease in cortical size is associated with a decrease in activity.

The medulla, in contrast to the cortex, does not appear to have changed significantly in size during the many generations of domestication undergone by the Norway rat, even though reduction in size of the cortex has given the medulla a greater proportion of the whole gland. On the other hand, in the Alexandrine rat we found medullary tissue in proportionately greater amounts than in the Norway. This fact may be related to the differences in behavior exhibited by these two species of rats.

While there was only a slight reduction in the weight of the adre-

nals of wild rats born in the laboratory, the F_1 generation of wild-domestic crosses had adrenals that were almost as small as those of the domestic. Thus, while in matings between wild rats many generations are needed to reduce the size of the adrenals down to the level of domestic rats, this result apparently can almost be achieved in crosses between wild and domestic rats in one generation.

It is probable that seasonal variations may be explained by the physiological reaction to the cold temperature of the winter months, the greater difficulty of obtaining food, and the general poorer health of street rats during the winter. Hartman (1931) and Selye (1936) have shown that short-term exposure to cold will cause adrenal hypertrophy. It has also been demonstrated that diet has a marked effect on the size of the adrenal (Blumenfeld, 1934; Ingle, 1945) and that unhealthy animals have larger adrenals than healthy ones (Grollman, 1936).

The islands of juvenile cortex seen in the wild Norways were similar in nature to those described in the domestic albino (Howard, 1938). The physiological significance of the band of connective tissue containing the juvenile cortical cells which surround the medulla of the wild Alexandrine is at present unknown.

SUMMARY

A comparative study was made of adrenal weights and cortico-medullary ratios in wild and domestic Norway rats and in wild Alexandrine rats.

In general the wild rats had proportionately much heavier adrenal glands than the domesticated form. Life under laboratory conditions from birth resulted in a slight reduction in adrenal size, but not as much as has occurred in laboratory rats after many generations of domestication. First generation crosses between wild and domestic Norways, however, had adrenals that weighed only slightly more than those of their domestic parents, indicating some genetic control of adrenal size.

The female Norway rats all had considerably heavier adrenals than the corresponding males. The sex difference in Alexandrines was not so marked.

A reduction in cortex size was found to be responsible for the difference in weight of the total glands, since the medulla was approximately the same size in all the rats.

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THE CHEMICAL HISTOLOGY AND CYTOLOGY OF THE PINEAL BODY AND NEUROHYPOPHYSIS[1]

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THE PRESENT PAPER gives an account of some cytological and histochemical features of the pineal body and neurohypophysis of the rhesus monkey. It concerns observations on the presence and distribution of nucleoproteins, lipids, glycogen, collagen, nerve fibers and alkaline phosphatase, explored by a variety of methods. It contains brief observations also in regard to autofluorescence and birefringence in these areas. Brief notes are also included on several cytological features of the neighboring adenohypophysis.

MATERIAL AND METHODS

The material consisted of the hypophyses and pineal bodies removed from 10 young, freshly killed rhesus monkeys (Macaca mulatta). The animals were between 1 and 3 years of age. Four of these monkeys were normal, whereas the others had previously been experimentally infected with malaria; the latter, however, showed no lesions of which we became aware in either the hypophysis or pineal body, the only noticeable abnormal feature being, in several, the presence of pigmented malarial parasites in some of the erythrocytes seen in the blood vessels.

Nucleoproteins were investigated with the basic dye methylene blue, by the Feulgen method and by means of ribonuclease. For these purposes blocks containing the pineal body and neurohypophysis were fixed in Zenker's fluid and 5μ paraffin sections were prepared. The deparaffinized sections were stained in eosin and methylene blue or methylene blue alone, while others were treated with a 0.1% solution of ribonuclease at 60° C for periods of $\frac{1}{2}$, $1\frac{1}{2}$ and 3 hours before staining them. This enzyme removes ribonucleoprotein, leaving the nuclear desoxyribonucleoprotein intact. For the specific purpose of demonstrating desoxyribonucleoprotein the Feulgen reaction was carried out on other sections; light green was used as a counterstain to the violet Feulgen reaction. For comparison of the cytology of nuclei and cytoplasm as revealed by these methods, still other sections were stained with Masson's triacid stain.

Lipids were investigated by staining with sudan black B. For this purpose the tissues were fixed in 10% neutral formalin and frozen sections were stained for 1 and 7 minutes in a 70% alcoholic solution of sudan black B.

Unstained frozen sections were examined under a polarizing microscope for the presence of birefringent material and in ultraviolet light for the pres-

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ence of natural fluorescence. The details of the fluorescence microscope utilized have been described elsewhere (Grafflin, '39).

Glycogen was sought for after fixation in a mixture of absolute alcohol, formalin and picric acid (Rossman's fluid). The sections were stained by the Bauer-Feulgen technique as well as by McManus' periodic acid method. Control sections were treated with saliva. The periodic acid technique, according to McManus ('46), demonstrates mucus, collagenous fibers, basement membranes and a variety of substances of unknown composition. We have found that it also stains glycogen very intensely. We have used Mcmanus' method ('46) in slightly modified form on sections cut from blocks of tissue fixed in Zenker's fluid or absolute alcohol, formalin and picric acid (Rossman's fluid). The deparaffinized sections were treated with a 1% solution of periodic acid for 5 minutes followed by Schiff's reagent for 15 minutes and subsequent rinsing in sulphurous acid. Whenever a counterstain seemed desirable, light green was used. The sections were then dehydrated in alcohols, cleared in xylol and mounted in balsam. With the periodic acid method, glycogen is told apart from mucus and other positively reacting substances by the use of control sections exposed to saliva.

Collagen was investigated by the use of Masson's triacid stain and Pap's ('29) ammoniacal silver method for reticulum. These are successful after any one of numerous fixatives, including Zenker's fluid, alcohol, formalin and picric acid (Rossman's fluid) and alcohol, formalin and acetic acid (Bodian's fixative no. 2).

Nerve fibers were stained in the neurohypophysis and pineal body by Bodian's protargol method. For this purpose the tissues were fixed in a mixture of alcohol, formalin and acetic acid (Bodian's fixative no. 2).

Alkaline phosphatase was studied by the method of Gomori ('41) as modified by Dempsey and Deane ('46). Blocks containing the pineal body and the neurohypophysis were fixed in chilled 80% alcohol. The deparaffinized sections were subjected to glycerophosphate, yeast nucleic acid and fructose diphosphate, used individually as substrates at pH 9.5 and incubated for 3, 24 and 72 hours. The sections were counterstained with paracarmine.

The pineal body is composed of parenchymal cells which are rather uniform in character throughout the organ. The neurohypophysis on the contrary consists of the neural lobe or infundibular process and the neural stalk or infundibulum. For purposes of the present investigations, unless otherwise stated, the descriptions apply to the cells and tissue characteristic of the infundibular process, no effort having been made to discern differences in the various parts of the neurohypophysis.

OBSERVATIONS

By the techniques employed, the parenchymal cells of the pineal body of young rhesus monkeys appear to be fairly uniform in character and of a single type in contrast to the several kinds of cells described in the pineal body of man. They have large, round nuclei and possess distinctly more cytoplasm than the pituicytes of the neurohypophysis. They are distinctly epithelioid in appearance in contrast to the fusiform and irregularly shaped pituicytes which

have small polymorphous nuclei ranging from round to flattened. The nuclei of the pineal cells are generally large and clear, and they possess several deeply stained, conspicuous nucleoli (figs. 1, 2 and 4). The nuclei of the pituicytes are smaller and more deeply stained and the nucleoli are inconspicuous (figs. 3, 13 and 18). Many of the nuclei of the pineal cells are bilobed, the two halves of the nuclei being separated by a deep narrow cleft or incisure which almost bisects the nucleus (fig. 4). A narrow bridge of protoplasm can usually be found connecting the two halves of the nucleus, although at times two separate nuclei are seemingly present. Occasionally one lobe appears to be much larger than the other and rarely 3 lobes are discernible. No similar configuration of the nuclei of the pituicytes has been encountered.

Nucleoproteins. Stained with methylene blue or eosin-methylene blue, the parenchymal cells of the rhesus monkey's pineal body possess a fairly abundant, moderately basophilic cytoplasm in which, with the oil immersion lens, minute particles and aggregations of basophilic material can be seen. In the neurohypophysis following the same procedure, little staining of the cytoplasm of the pituicytes can be made out; at most a little basophilic material is visible close to the nucleus. Although the pituicytes are sparsely provided with cytoplasmic basophilia, the surrounding neuropil shows a mild degree of basophilic staining. In the case of the application of eosin and methylene blue this manifests itself in a purplish shade.

When sections of the pineal gland are treated with ribonuclease before staining them with methylene blue or eosin-methylene blue, the blue-staining cytoplasmic component of the parenchymal cells is completely abolished (figs. 15 and 16). This loss of staining indicates that ribonucleoprotein is responsible for the cytoplasmic basophilia. The staining of the nuclei and nucleoli, on the contrary, is not very perceptibly diminished. After the Feulgen technique, the nuclear chromatin and a number of the nucleoli are stained intensely violet, a reaction which is regarded as being specific for desoxyribonucleoprotein. In addition to the several conspicuously violet nucleoli (karyosomes), a nucleolus can often be identified which is Feulgen negative and is tinged by the light green used as counterstain; this we interpret as being a nucleolus containing ribonucleoprotein.

The mild basophilia of the neurohypophysis which involves the neuropil and the cytoplasm of a few of the pituicytes is also prevented by ribonuclease (figs. 13 and 14). In other parts of the brain, especially in the grey matter, a similar staining of the neuropil with methylene blue is encountered.

Lipids. The staining of individual cells as observed in frozen sections of the pineal body and the infundibular process of the hypophysis is illustrated in figures 5 and 6. The parenchymal cells of the pineal body are rather uniform in appearance, exhibiting delicately

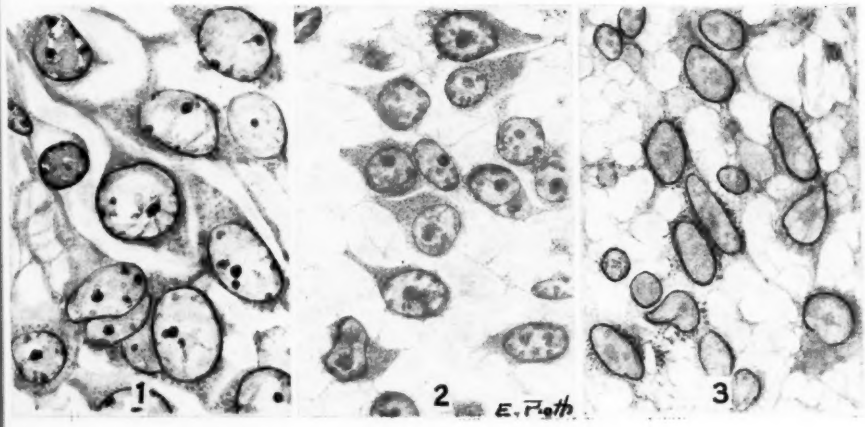

PLATE 1

FIG. 1. Pineal body of a young rhesus monkey. Fixation in Zenker's fluid followed by staining in eosin-methylene blue. Observe the basophilic staining of the cytoplasm of the parenchymal cells. ×10 oc., ×90 obj.

FIG. 2. Pineal body of a young rhesus monkey. Fixation in cold 80% alcohol followed by staining for alkaline phosphatase. Yeast nucleic acid utilized as substrate; section incubated for 72 hours at pH 9.5. Counterstained with paracarmine. ×7 oc., ×90 obj.

FIG. 3. Infundibular lobe of the hypophysis of a young rhesus monkey. Fixation in cold 80% alcohol, followed by staining for alkaline phosphatase. Fructose diphosphate utilized as substrate; section incubated for 72 hours at pH 9.5. Counterstained with paracarmine. ×7 oc., ×90 obj.

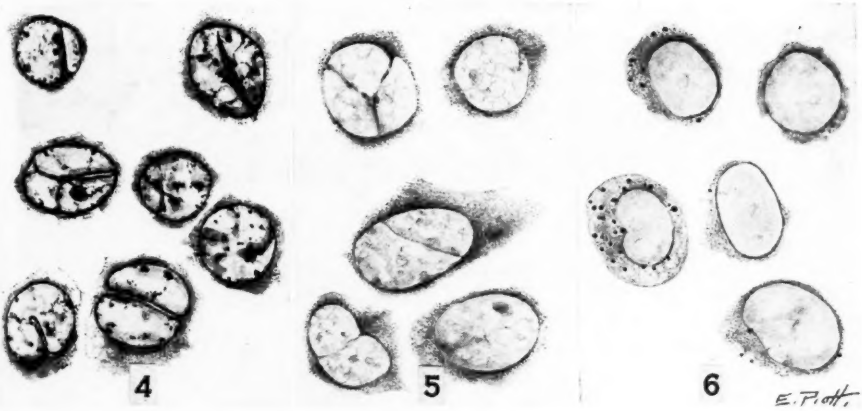

PLATE 2

Explanation of figures on Plate 2

FIG. 4. Pineal body of young rhesus monkey. Fixation in a mixture of absolute alcohol, formalin and picric acid, followed by Masson's stain. Selected, individual cells have been drawn to illustrate the morphology of the nucleus. Many nuclei are bilobed as a result of a deep narrow indentation on one side. ×10 oc., ×90 obj.

FIG. 5. Pineal body of a young rhesus monkey. Fixation in 10% neutral formalin, followed by staining of a frozen section for 7 minutes in sudan black B. Selected parenchymal cells showing the cytoplasm filled with extremely delicate, grey sudanophilic material. ×10 oc., ×90 obj.

FIG. 6. Infundibular lobe of the hypophysis of a young rhesus monkey. Fixation in 10% neutral formalin, followed by staining of a frozen section for 7 minutes in sudan black B. Selected parenchymal cells showing delicate, black sudanophilic particles in their cytoplasm. ×10 cc., ×90 obj.

stippled grayish cytoplasm surrounding the nuclei (fig. 5). The parenchymal cells of the infundibular process, on the contrary, contain small, black sudanophilic bodies which range from none up to 20 or 30 in number (fig. 6).

Birefringence and autofluorescence. Frozen sections of the pineal body and neurohypophysis were examined for these properties. Excepting the collagenous fibers around the blood vessels which are always strongly birefringent, no doubly refractile substances are encountered in either the pineal body or neurohypophysis.

A very faint diffuse sheen is visible throughout the sections, but specific autofluorescence is not observed in the parenchymal cells. The elastic interna of the blood vessels fluoresces a luminous whitish blue wherever cerebral arterioles are seen.

In regard to these properties, the cells of the anterior lobe and pars intermedia of the hypophysis are negative throughout.

Glycogen. Following appropriate fixation of the pineal body and neurohypophysis in a mixture of absolute alcohol, formalin and picric acid followed by the Bauer-Feulgen stain for glycogen, cells and tissue elements of these organs are completely negative.

Upon applying McManus' periodic acid technique following the same fixative, several interesting observations can be made. The collagenous reticulum of both the pineal body and the neurohypophysis is stained a deep pinkish red which is not altered by previous exposure of the sections to saliva. In addition, some of the parenchymal cells of the pineal body show distinct red cytoplasmic staining. These are generally cells containing a relatively large amount of cytoplasm which is filled with minute red granules which are often arranged to form short rods or threads. In some few of the cells the reddish substance seems to extend out a slight distance into cytoplasmic processes of the cells. Other, more plentiful cells, exhibiting less cytoplasm, contain the reddish material only immediately around the nucleus, while still other cells which are quite numerous, contain none of these reddish elements whatsoever. Staining of the red substance is prevented by the use of saliva and consequently it must be attributed to the presence of glycogen.

It is interesting that glycogen does not become evident after the Bauer-Feulgen method but is visible only after the use of the periodic acid technique. The explanation which we have to offer is that the periodic acid procedure is very much more delicate than any of the previous methods for glycogen. We base this conclusion upon repeated comparisons of the results of the periodic acid method in a variety of tissues with Best's stain and the Bauer-Feulgen reaction as well as with the silver-nitrate method of Mitchell and Wislocki ('44). The periodic acid invariably brings out more glycogen than the latter and in the present instance it differentiates very delicate amounts of this substance where none is demonstrable at all by other histological means.

Another interesting feature of the periodic acid method is that it differentiates parenchymal cells in the pineal body, which contain glycogen, from others which contain little or none. This finding suggests that the parenchymal cells may be of different types or at least that they may be in different stages of functional activity. In the infundibular lobe of the hypophysis no glycogen comparable to that observed in the pineal body is encountered. In the adenohypophysis

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Explanation of figures on Plate 3

FIG. 7. Pineal body of a young rhesus monkey. Fixation in cold 80% alcohol, followed by staining for alkaline phosphatase. Fructose diphosphate used as substrate and section incubated for 72 hours at pH 9.5. ×240.

FIG. 8. Infundibular lobe of the hypophysis from the same monkey. Fixed and stained in the same way (fructose diphosphate at pH 9.5). Compare with field of the same seen at higher magnification in figure 3. ×240.

FIG. 9. Pineal body prepared with yeast nucleic acid as substrate and incubated for 72 hours at pH 9.5. Compare with field of the same seen in figure 2 at higher magnification. ×240.

FIG. 10. Infundibular lobe, the same as above, similarly stained with yeast nucleic acid as substrate at pH 9.5. ×240.

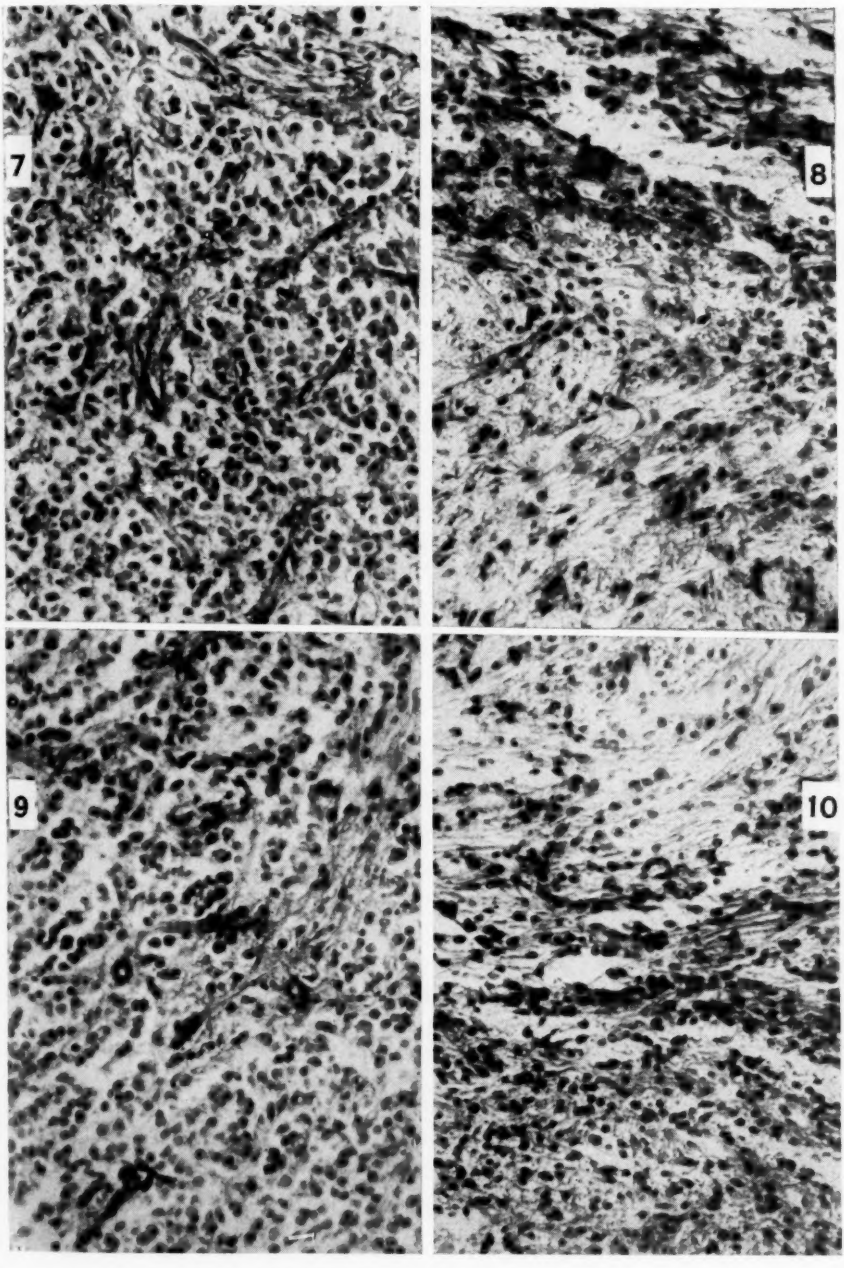

PLATE 3

no specific staining of the epithelial cells is noticeable by the periodic acid technique as we have used it. However, wherever colloid cysts occur in the adenohypophysis, the colloid filling these small vesicles reacts positively by both the Bauer-Feulgen and periodic acid techniques and in neither case is the staining prevented by saliva. The staining of this colloid by the Bauer-Feulgen method has been previously noticed by Wallraff and Beckert ('39).

Collagen. The staining of collagen and of collagenous reticulum in the pineal body and neurohypophysis has been observed in Masson preparations as well as in sections impregnated with silver by Pap's method ('29). The blood vessels penetrating the interior of these organs are surrounded by conspicuous sheaths or networks of reticular fibers (figs. 11 and 12). The vessels supplying these areas contrast sharply with the cerebral vessels penetrating the rest of the brain, which are not accompanied by any such elaborate and abundant connective tissue sheaths. The presence and permeability of these sheaths account apparently for the staining of the neurohypophysis, pineal body and other small areas of the brain by vital dyes such as trypan blue (Wislocki and King, '36; King, '39).

Nerve fibers. For the demonstration of nerve fibers the pineal body and neurohypophysis were stained by Bodian's protargol method following fixation of the tissues in Bodian's fixative no. 2. By this technique delicate nerve fibers are brought out in each area (figs. 17 and 18). In the case of the pineal body the fibers occur mainly on the outskirts of the parenchymal cords in association with the reticular networks subdividing the gland, as will be appreciated by comparing figures 11 and 17. The nuclei of the parenchymal cells are also quite intensely silvered. In the infundibular lobe, on the contrary, the fibers appear to be smaller, much more numerous and to run every which way without particular reference to any recurring pattern of the parenchymal cells or to the collagenous strands subdividing the infundibular process (cf. figs. 12 and 18).

Alkaline phosphatase. Both pineal body and neurohypophysis of the rhesus monkey contain alkaline phosphatase when sections are incubated at pH 9.5 for 24 hours or longer; after 3 hours' incubation

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Explanation of figures on Plate 4

FIG. 11. Pineal body of a young rhesus monkey, stained to show the argyrophilic collagenous reticulum. Fixation in a mixture of absolute alcohol, formalin and picric acid, followed by Pap's alkaline silver method for reticulum. ×140.

FIG. 12. The infundibular lobe of the hypophysis of the same monkey, similarly fixed and stained to show the argyrophilic collagenous reticulum. ×140.

FIG. 13. The infundibular lobe of a young rhesus monkey. Fixation in Zenker's fluid followed by staining with eosin-methylene blue. Observe the relatively faint staining of cell cytoplasm and neuropil. ×220.

FIG. 14. The infundibular lobe of the same monkey, fixed as before. The section was exposed to a solution of ribonuclease before staining with eosin-methylene blue. By comparison with figure 13, it will be observed that little besides the nuclei is visible after treatment with ribonuclease. ×220.

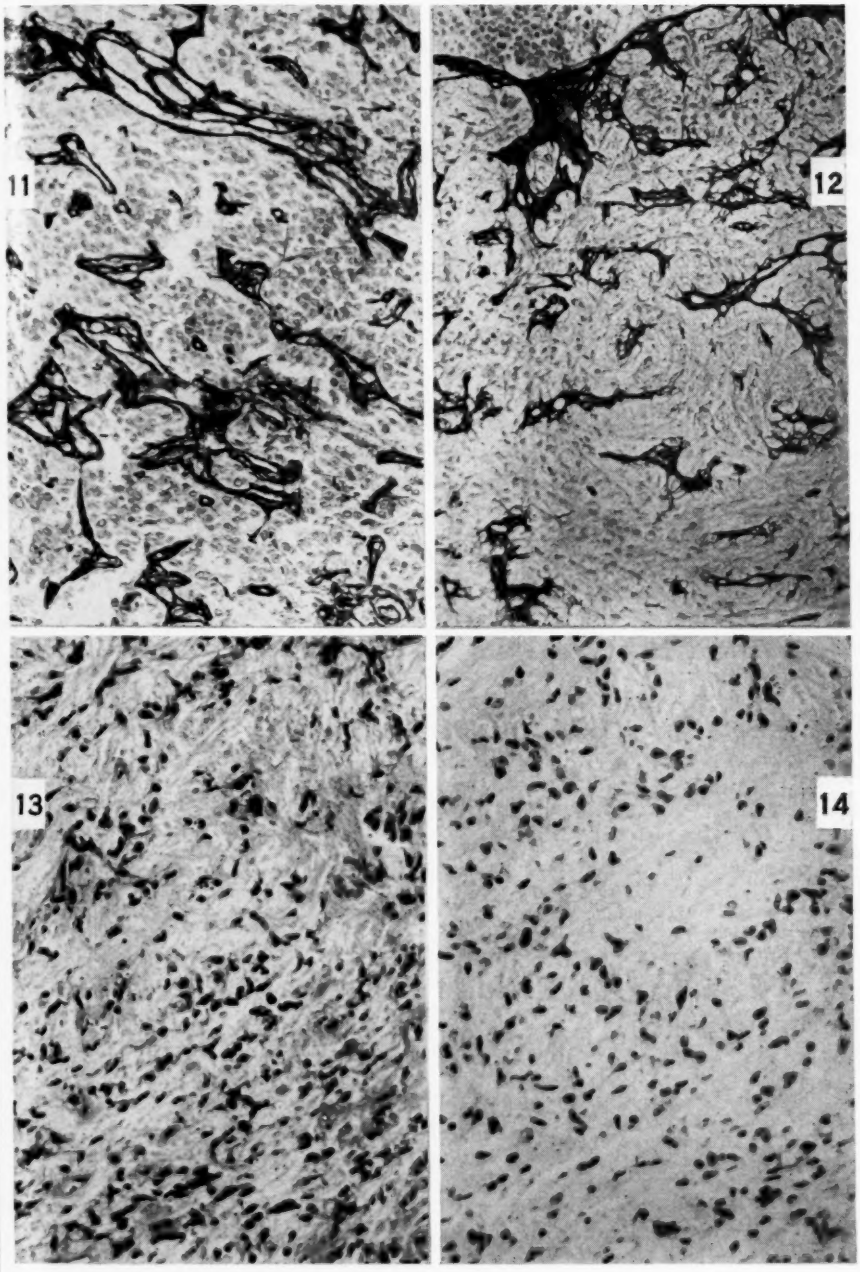

PLATE 4

the reactions are so very slight that they might well be designated as negative. The pineal body reacts more uniformly and strongly than the neurohypophysis; the reaction in the latter is more intense in the center of the infundibular lobe than in the peripheral part. Of the 3 substrates used, the strongest reaction is obtained with fructose diphosphate and the next best with nucleic acid, while with glycerophosphate the least activity is demonstrable.

Parenchymal cells, blood vessels and the perivascular sheaths of the pineal body give pronounced reactions (figs. 2, 7 and 9). The reaction in the parenchymal cells involves both the cytoplasm and nuclei and extends out a short distance into the cell processes. With glycerophosphate the reaction is least intense. With nucleic acid the walls of the blood vessels are especially deeply stained, while the reaction of the perivascular sheaths is less intense; with fructose diphosphate vessel walls as well as the perivascular tissue are deeply colored.

The parenchymal cells in the interior of the neurohypophysis exhibit intense reactions at pH 9.5 after 24 hours or more incubation with fructose diphosphate and nucleic acid as substrates (figs. 3, 8 and 10), the color with the former being most concentrated in the cytoplasm and with the latter about equally distributed between cytoplasm and nucleus. The cortical zone of the infundibular lobe is much less intensely colored, and with glycerophosphate there is practically no reaction anywhere in the neurohypophysis. The penetrating blood vessels are less deeply and uniformly stained than in the pineal body. The neuropil, in the center of the infundibular process, comprising fibrillar and punctate interstitial material, is variably deeply stained.

It will be recalled that the pituicytes are believed to be related to neuroglial cells (Bucy, '32) more particularly to astrocytes derived from spongioblasts (Griffiths, '39). In view of this, it is of interest that the pituicytes contain alkaline phosphatase, whereas the neuroglial

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Explanation of figures on Plate 5

FIG. 15. Pineal body of young rhesus monkey. Fixation in Zenker's fluid, followed by staining with eosin-methylene blue. Observe the size and staining of the parenchymal cells and compare with figure 1 which presents the appearance of a field seen under higher magnification. ×240.

FIG. 16. Pineal body of the same monkey, fixed as before. The section was exposed to a solution of ribonuclease, before staining with eosin and methylene. Observe that the cytoplasmic basophilia (cf. figs 1 and 15) has been totally removed from the parenchymal cells by the enzyme leaving only the nuclei stained. The nucleoli have also retained their basophilia. ×240.

FIG. 17. Pineal body of another monkey fixed in a mixture of absolute alcohol, formalin and acetic acid (Bodian's fixative no. 2) and stained by Bodian's protargol method. Observe the fields of parenchymal cells surrounded by relatively acellular zones in which silvered nerve fibers are visible. The nuclei of the parenchymal cells are quite intensely silvered. Compare the size and number of the nerve fibers with the dense network of reticular fibers surrounding the fields of parenchyma (fig. 11). ×240.

FIG. 18. The infundibular lobe of the hypophysis of the same monkey, similarly stained by Bodian's protargol method. Note the greater wealth of nerve fibers and the smaller, less conspicuous cells than in the pineal body. Compare this preparation with figure 12 in which the argyrophil collagenous reticulum has been brought out. ×240.

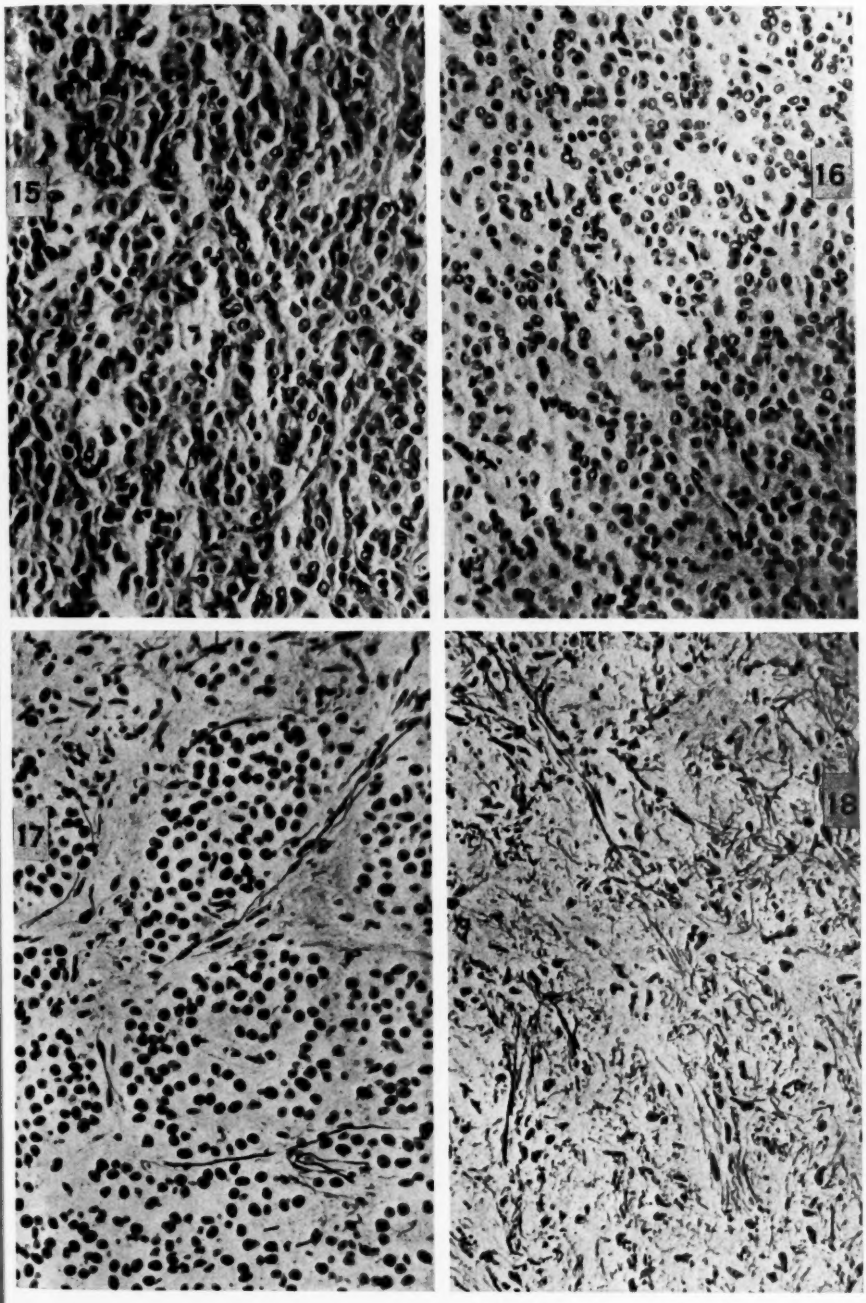

PLATE 5

cells, as observed throughout the cerebral convolutions in both grey and while matter, are very generally devoid of alkaline phosphatase in either cytoplasm or nuclei. In the cerebral grey matter we have observed repeatedly that alkaline phosphatase is most intense and most readily demonstrable in the blood vessels, that it occurs with great constancy in the neuropil as well as in the perikaryon and nuclei of the neurons, but that it is very nearly absent in the nuclei and bodies of the glial cells.

Incidentally to studying the neurohypophysis, the adenohypophysis has been examined. In general the latter is negative for alkaline phosphatase, although slight enzymatic activity is revealed in sections incubated for 72 hours utilizing fructose diphosphate and glycerophosphate. This little is present in quite irregular patches in the parenchymal cells of the pars distalis and pars intermedia. None has been encountered in the cells of the pars tuberalis, nor has any been seen in the walls of the blood vessels of any portion of the adenohypophysis. The absence of phosphatase in these blood vessels distinguishes them from the vessels penetrating the brain, the walls of which contain large amounts of alkaline phosphatase.

DISCUSSION

The present study reveals significant differences as well as some similarities in the cytology and histochemistry of the pineal body and neurohypophysis of the rhesus monkey. These will be briefly summarized and discussed.

Nucleoproteins. The parenchymal cells of the pineal body contain an appreciable amount of cytoplasmic ribonucleoprotein besides large and frequently bilobed, nuclei inside which are conspicuous chromatin bodies. The presence of large, chromatin-rich nuclei in combination with the basophilic cytoplasm suggests that the cells are engaged in active nucleoprotein synthesis. In possessing marked basophilia of both nucleus and cytoplasm they differ from the common neuroglial cells of brain tissue.

Some of the nuclei of the human pineal body contain conspicuous spherical inclusions (cf. del Rio-Hortega, '32) discovered by Dimitrova ('01). These bodies she regarded as liberating secretion, because at times, according to her, they approach the nuclear membrane, empty their contents into the cytoplasm and disappear. Basophilic granules which are said to be discharged into the cytoplasm have been described within them. Yet, Achúcarro and Sacristán ('12) have shown that the spheres of Dimitrova, instead of being nuclear, consist of cytoplasm enclosed within folds and inpocketings of the nuclei. Del Rio-Hortega ('32) believes, too, that the formation of the inclusions is initiated by depressions, wrinkles and folds of the nuclei. Besides the several authors who ascribe the role of nuclear secretion to these

structures, others have regarded them as representing regressive changes in the parenchymal cells.

In the pineal body of young rhesus monkeys we have not encountered any similar spherical inclusions. In view, however, of the deep incisure or cleft which exists in many cells between the lobes of the nucleus one can readily visualize how a pocket of cytoplasm might become sequestered and possibly completely snared off by progressive infolding of the nucleus.

To regard the spheres of Dimitrova as a part of the nucleus which discharges periodically into the cytoplasm is an interesting thought in view of the recent demonstration of Caspersson ('39) of the release of ribonucleoprotein into the cytoplasm by cell nuclei through the activity of the nucleoli. It seems likely, however, as del Rio-Hortega and others maintain, that these structures are cytoplasmic rather than nuclear. Nevertheless, the peculiar bilobed condition of many of the parenchymal cells resulting in an increase in nuclear surface might be a sign of increased nuclear activity in Caspersson's sense. And, further, if the nucleus were to liberate ribonucleoprotein into the nuclear cleft, its accumulation in the cytoplasm there might conceivably result in the formation of a basophilic retention vacuole identical with the spheres of Dimitrova. So that looked at in a somewhat different light, through information on nuclear activity recently supplied by Caspersson, one might suggest that the spheres of Dimitrova represent possibly nuclear secretion which has been discharged into the cytoplasm of the nuclear cleft.

In contrast to the parenchymal cells of the pineal body, the pituicytes of the neurohypophysis contain exceedingly little basophilic material in their cytoplasm and none of their nuclei exhibit distinctive clefts.

Lipids. Ignoring the faint grey stippling present in the background, pituicytes differ from the parenchymal cells of the pineal body in possessing definite lipid particles stainable by sudan black B. These bodies in the pituicytes of the monkey would seem to be homologous with similar sudanophilic lipid material discovered by Gersh ('39) in the pituicytes of a variety of mammals. Of the rhesus monkey, he speaks only of examination of a fresh, free-hand slice of the neurohypophysis in which the parenchymatous cells appeared as clear nuclei surrounded at times by fine, refractile granules. It seems likely that these granules would be homologous with the sudanophilic ones described in the present investigation. Gersh has suggested that this lipid is related to the production of the antidiuretic factor by the neurohypophysis, increasing in amount in the pituicytes when water-intake is restricted, but Hickey, Hare and Hare ('41) have denied such a relation. Gersh points out that cells bearing lipid material of this character are present only in the neurohypophysis among the

regions of the brain which he examined. He remarks that no cells of this type are encountered in either the area postrema or the preoptic area, regions which, in respect to blood supply and some other histological features (Wislocki and King, '36), resemble the neurohypophysis. To Gersh's examples we can now add the cells of the pineal body as bearing no resemblance to pituicytes in regard to the possession of lipid particles.

Glycogen. The parenchymal cells of the pineal body contain saliva-soluble material in their cytoplasm, presumably glycogen, which is stainable by McManus' periodic acid method, but not demonstrable by the Bauer-Feulgen procedure. The periodic acid technique has revealed in our experience much more glycogen at known sites of its occurrence than any of the current methods for the staining of this substance. However, in the pineal body, small amounts of glycogen are revealed suddenly by this technique where none whatsoever is visible by other methods.

This is the only part of the brain among the areas examined in which periodic acid reveals any glycogen. None was encountered in the cells of the neurohypophysis, nor was any observed in neurons or neuroglial cells in white and grey matter in the hypothalamus or in areas adjacent to the pineal body.

Alkaline phosphatase. Phosphatases are enzymes capable of splitting organic phosphates, for example, glycerophosphate, hexose diphosphate, nucleic acid and lecithin. They play important roles in the metabolism of carbohydrates, lipids and nucleoproteins. In the present investigation alkaline phosphatase has been found within the parenchymal cells of both the pineal body and the neurohypophysis. In the former it is associated with the presence of cytoplasmic nucleoprotein and glycogen, and in the latter with lipids which bear a possible relationship to the specific functioning of the neurohypophysis. It is perhaps worthy of remark that, utilizing radioactive phosphorus injected intraperitoneally, Borell and Örström ('47) have found a high phosphate turnover in the pineal body of a variety of animals.

Concluding comment. The cytological evidence presented in this paper indicates that the cells of the pineal body and neurohypophysis differ very markedly, suggesting that their functional activities are probably very dissimilar.

Although the parenchymal cells differ in important cytological features, the similarity of the neurohypophysis and the pineal body in several histological respects is very close. It has been demonstrated (Wislocki and King, '36; King, '39) that the neurohypophysis, pineal body, area postrema and supraoptic crest have certain histological features in common which we shall briefly cite. (1) Their intrinsic blood supply is rich and the vessels are surrounded by conspicuous vascular sheaths composed of fibrous reticulum (cf. figs. 11 and 12). Both the pattern of the vessels and their connective tissue sheaths

distinguish them from the small cerebral vessels in general (cf. Cammermeyer, '44). This has been perhaps most conclusively demonstrated in the opossum where the neurohypophysis and area postrema are vascularized by plexiform blood vessels rich in connective tissue, whereas the rest of the brain is supplied by individual slender end-arteries and non-anastomotic, thin-walled capillary loops peculiar to the cerebral circulation of the opossum (Wislocki, '40). (2) These organs possess also in common the property of staining with vital azo dyes, a peculiarity which distinguishes them from the bulk of the brain which does not stain vitally because of the interposition of the "blood-brain" barrier (King, '39). (3) Finally, it might be pointed out that these structures are all in close proximity to the pia arachnoid, some, such as the neurohypophysis and pineal body, virtually protruding into the periaxial mesoderm. This consideration indicates again the peculiar association of these parts of the brain with connective tissue.

Because of these various properties, King has remarked that these structures "are in the nervous system but not of it." Closer perhaps to actuality would be to say that their specialized parenchymal cells are of the central nervous system, being related to and differentiated from ordinary neuroglia, but that the arrangement of their blood vessels and connective tissue as well as their permeability to vital dyes allies them to mesenchymal organs rather than to the brain. In the latter respect they are more like the peripheral nervous system or its derivative the adrenal medulla.

SUMMARY

The present cytological and histochemical investigations of the pineal body and neurohypophysis of the rhesus monkey reveal that the parenchymal cells of these two structures differ in a number of respects. The parenchymal cells of the pineal body are characterized by their large, chromatin-rich, lobed nuclei and the presence in their cytoplasm of ribonucleoprotein, alkaline phosphatase and traces of glycogen. The pituicytes, on the contrary, display lipid droplets, alkaline phosphatase, mere traces of ribonucleoprotein and no glycogen. These characteristics suggest differences in function in the two organs. Although the parenchymal cells of the pineal body and neurohypophysis are believed to be related to neuroglia, they have diverged from them to become separately specialized, judging from their histochemical properties.

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NOTES AND COMMENTS

CALCIUM DEPOSITION IN THE FEMORA OF CHICK EMBRYOS AFTER THE INJECTION OF ESTRADIOL BENZOATE[1]

It has been observed by many investigators that the injection of estradiol benzoate into various species of birds and mammals produces hypercalcification of the femora. The immature birds of some breeds show a more vigorous response than the adults (Day and Follis, 1941). However, in New Hampshire Red immature cockerels, the skeletal effects of estrogen are comparatively slight (Salomon, Gabrio, Reinhard, and Silberberg, 1947) while extensive hyperossification of the long bones occurs in the mature cock of this breed in response to estrogen (Landauer, Pfeiffer, Gardner, and Man, 1939). These and other variations suggest than an age factor partially determines the estrogenic response.

Fell and Robison (1934) studied histologically the development of the calcifying mechanism in chick embryos and reported that the osteoid tissue rapidly acquires a high degree of calcifiability. From about the 12th to the 21st days of incubation, fairly active bone formation is going on, and a relatively greater amount of calcium is available for bone calcification than in the adult stage.

From the foregoing considerations, it seemed of interest to investigate whether or not the hyperossification phenomenon could be produced at a very early stage of development and if estrogen could serve as an impetus to the calcifying mechanism or as a possible factor in the process. Thus, this experiment, which is preliminary in nature, was set up to determine if the injection of estradiol into the hen's egg prior to and after sex differentiation would result in hyperossification of the femora as indicated by increased calcium content.

MATERIALS AND METHODS

Fertilized eggs of New Hampshire Red Breed were used in this experiment. Various doses of alpha estradiol benzoate[2] were injected into some of the embryos under sterile conditions, and the eggs were returned to the incubator until the 15th or 18th day. At the time of sacrifice, the reproductive organs of the injected embryos and the controls were examined macroscopically.

Sixty-seven embryos were used in this investigation, and the 134 femora were analyzed for calcium content at the determined intervals. Ashing of the bones, prior to the determination of calcium, was carried out with concentrated nitric acid under an overhead heater (Nims and Horwitt, 1936)

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[2] The alpha estradiol benzoate (Progynon B) was generously supplied by the Schering Corporation through the courtesy of Mr. L. H. Cramblet.

and completed in a muffle furnace at 500°C. The ash was dissolved in 0.04 N hydrochloric acid and diluted with redistilled water. An aliquot was taken for calcium analysis following the titrimetric procedure of Salomon, Gabrio, and Smith (1946).

The chick embryos were divided into three groups:

Group I. Untreated Embryos. Since we were unable to find in the literature data pertaining to the calcium content of the femora of chick embryos at various stages of incubation, it was thought desirable to secure such information if possible. The femora of normal, untreated embryos and chicks were analyzed for calcium content at the following stages of development: 15 days incubation (7 females, 4 males), 18 days incubation (4 females, 6 males), and 1-day hatched chicks (3 females, 3 males).

Group II. Estrogen Injected at 10th Day of Incubation and Controls. Twelve embryos were injected on the 10th day of incubation with 1250 I.U. estradiol benzoate (2 females, 3 males), and 2500 I.U. estradiol benzoate (3 females, 4 males) respectively. Two female and 3 male uninjected embryos served as controls. All were sacrificed on the 18th day of incubation.

Group III. Estrogen Injected at 1st Day of Incubation and Controls. Sixteen 1-day embryos were injected with 1000 I.U. (4 females, 2 males), 2000 I.U. (2 females, 4 males), and 2500 I.U. estradiol benzoate (2 females, 2 males) respectively. Three female and 4 male uninjected embryos served as controls. All were sacrificed on the 18th day of incubation.

RESULTS AND COMMENTS

The analytical results obtained are represented in Tables I, II, and III. The data of Table I and the control data of Tables II and III indicate that no definite quantitative calcium content of the femora may be ascribed to a

TABLE I. CALCIUM CONTENT OF FEMORA OF UNTREATED EMBRYOS AND CHICKS

Group I	No. of femora	Dry wt. (mg.)		Mg. Ca.		% Ca. to dry wt.	
		Mean	σ	Mean	σ	Mean	σ
15-day Embryos	22	32.0	6.04	7.49	.930	24.03	4.345
18-day Embryos (Series A)	12	26.3	1.25	6.76	1.039	25.83	4.523
18-day Embryos (Series B)	8	32.2	4.42	12.14	1.322	37.97	4.029
1-day Hatched	12	58.6	4.93	11.53	1.222	19.69	1.714

particular incubation stage. For example, in the 18-day embryos of Table I, the eggs of Series A and the eggs of Series B were obtained from the same farm at different times, but analyses of the femora show great differences in calcium content. In the 18-day control embryos of Tables II and III still further variations are noted, so that there are four different mean values of calcium content of 18-day femora: 6.76 mg., 12.14 mg., 3.39 mg., and 3.03 mg. calcium respectively. The latter two mean values which are similar may be accounted for by the fact that the embryos of Tables II and III were hatched from the same batch of eggs. The many variable components such

TABLE II. CALCIUM CONTENT OF FEMORA OF 18-DAY EMBRYOS INJECTED WITH ESTRADIOL BENZOATE ON 10TH DAY OF INCUBATION

Group II	No. of femora	Dry wt. (mg.)		Mg. Ca.		% Ca. to dry wt.	
		Mean	σ	Mean	σ	Mean	σ
1250 I.U. estradiol benzoate	10	16.7	3.50	3.36	.856	20.04	2.559
2500 I.U. estradiol benzoate	14	18.5	2.63	4.00	.540	21.87	3.534
Control	10	18.1	3.57	3.39	.645	18.96	3.032

as hen's ration, pre-incubation temperature to which the eggs were exposed, and the original weights of the eggs result in the extreme variations. However, the standard deviation of each group is small in comparison to the deviations between different groups. It is thus impossible to obtain satisfactory normal data on the calcium content of chick femora at different stages of incubation unless one considers a single series of embryos from one batch of eggs. Therefore, in this study of bone in response to estrogenic treatment in chick embryos, all of the groups were taken from a single batch of eggs.

The data of Table II indicate that neither 1250 I.U. nor 2500 I.U. estradiol benzoate injected on the 10th day of incubation caused increased amounts of calcium to be deposited in the embryonic bones by the 18th day of incubation. Since the standard deviations are comparatively large, the slight increases which may be noted in the treated embryos are without significance. Although Dantchakoff (1935) has reported that estrogen injection into 10-day embryos causes a slight degree of sex reversal in the males in some cases, we did not observe this phenomenon in any of our embryos injected on the 10th day, nor did Wolff and Ginglinger (1935) find intersexuality after the injection of folliculine on the 12th day of incubation.

From the data of Table III it appears that even though the embryos were exposed to the hormone for 17 days at the most, no significant change in calcium content of the femora can be noted in comparison to the controls. The injected females of this group did not show structural alterations of the gonadal system in response to estrogen except for one female who received

TABLE III. CALCIUM CONTENT OF FEMORA OF 18-DAY EMBRYOS INJECTED WITH ESTRADIOL BENZOATE ON 1ST DAY OF INCUBATION

Group III	No. of femora	Dry wt. (mg.)		Mg. Ca.		% Ca. to dry wt.	
		Mean	σ	Mean	σ	Mean	σ
1000 I.U. estradiol benzoate	12	19.7	1.65	3.25	.286	16.53	1.043
2000 I.U. estradiol benzoate	12	19.0	1.59	2.97	.234	15.85	2.854
2500 I.U. estradiol benzoate	8	19.5	2.24	2.94	.335	15.06	.153
Control	14	21.9	1.45	3.03	.348	13.85	1.680

2500 I.U. estradiol and who exhibited a slightly lengthened right oviduct. However, in one of the males receiving 2500 I.U. estradiol, the left testis was flattened, larger, and composed of ovarian and testicular tissue (ovotestis). Remnants of oviducts persisted on both sides. In some of the other male embryos the gonadal changes were less pronounced. Our morphological findings then are similar to those of Willier et al. (1937).

Thus, while the injection of certain doses of estradiol benzoate into 1-day chick embryos affects the reproductive system by causing degrees of intersexuality, hypercalcification of the femora cannot be produced by this hormonal impetus. Although the calcifying mechanism of older birds is sensitive to estrogen, it seems that in the embryonic period a different process is in operation.

SUMMARY

Calcium analyses of the femora of untreated 15-day and 18-day embryos and 1-day chicks indicate that an acceptable series of normal chemical data cannot be obtained unless embryos from a single batch of eggs, hatched and kept under identical conditions, are used.

Calcium analyses of the femora of 18-day embryos, injected on the 10th day of incubation with 1250 I.U. and 2500 I.U. estradiol benzoate, indicate that hypercalcification of the femora does not occur in response to estrogen.

Calcium analyses of the femora of 18-day embryos, injected on the first day of incubation with 1000 I.U., 2000 I.U. and 2500 I.U. estradiol benzoate, also indicate that estrogen-induced hyperossification is not produced. However, some morphological changes in the gonadal system of the males were noted.

It is suggested that the response of the calcifying mechanism to estrogen is different in the embryonic period of chicks than the response at later stages of development.

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